PATENT COOPERATION TREA

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
369.100		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 99/ 18016	13/08/1999	14/08/1998
Applicant		
CHIRON CORPORATION et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. -

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/ 18016

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 37-40 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/37 C07K14/205 A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 14564 A (LUDMERER STEVEN ;MERCK & CO INC (US)) 9 April 1998 (1998-04-09) examples 5,6 ---	1-4,6,10
X	WO 96 05293 A (UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION ;BARALLE FRANCESCO) 22 February 1996 (1996-02-22) page 13, line 25 -page 17, line 10 --- -/--	1-3

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 February 2000	28/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Cupido, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHANG C ET AL: "Phenotypic mixing between different hepadnavirus nucleocapsid proteins reveals C protein dimerization to be cis preferential" JOURNAL OF VIROLOGY, vol. 68, no. 8, August 1994 (1994-08), pages 5225-5231, XP002130379 AMERICAN SOCIETY FOR MICROBIOLOGY US cited in the application page 5228, left-hand column, last paragraph -page 5229, right-hand column, paragraph 1 ---	1,2
A	HOFMANN K J ET AL: "Sequence conservation within the major capsid protein of human papillomavirus (HPV) type 18 and formation of HPV-18 virus-like particles in Saccharomyces cerevisiae" JOURNAL OF GENERAL VIROLOGY,GB,SOCIETY FOR GENERAL MICROBIOLOGY, READING, vol. 77, no. 3, March 1996 (1996-03), pages 465-468, XP000604634 ISSN: 0022-1317 the whole document -----	41-51

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9814[redacted] A	09-04-1998	EP 0956549 A	17-11-1999
		US 5922588 A	13-07-1999
WO 9605293 A	22-02-1996	AT 402898 B	25-09-1997
		AT 154594 A	15-02-1997
		AU 3257495 A	07-03-1996
		EP 0775198 A	28-05-1997
		US 5932426 A	03-08-1999

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 99/18016

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/37 C07K14/205 A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 14564 A (LUDMERER STEVEN ;MERCK & CO INC (US)) 9 April 1998 (1998-04-09) examples 5,6	1-4,6,10
X	WO 96 05293 A (UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION ;BARALLE FRANCESCO) 22 February 1996 (1996-02-22) page 13, line 25 -page 17, line 10	1-3

-/—

[X] Further documents are listed in the continuation of box C.

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 February 2000	28/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Cupido, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/US 99/18016

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	CHANG C ET AL: "Phenotypic mixing between different hepadnavirus nucleocapsid proteins reveals C protein dimerization to be cis preferential" JOURNAL OF VIROLOGY, vol. 68, no. 8, August 1994 (1994-08), pages 5225-5231, XP002130379 AMERICAN SOCIETY FOR MICROBIOLOGY US cited in the application page 5228, left-hand column, last paragraph -page 5229, right-hand column, paragraph 1	1,2
A	HOFMANN K J ET AL: "Sequence conservation within the major capsid protein of human papillomavirus (HPV) type 18 and formation of HPV-18 virus-like particles in Saccharomyces cerevisiae" JOURNAL OF GENERAL VIROLOGY,GB,SOCIETY FOR GENERAL MICROBIOLOGY, READING, vol. 77, no. 3, March 1996 (1996-03), pages 465-468, XP000604634 ISSN: 0022-1317 the whole document	41-51

INTERNATIONAL SEARCH REPORT

Ir.. ..national application No.
PCT/US 99/ 18016

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 37-40 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern ıal Application No
PCT/US 99/18016

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9814564 A	09-04-1998	EP 0956349 A	17-11-1999
		US 5922588 A	13-07-1999
WO 9605293 A	22-02-1996	AT 402898 B	25-09-1997
		AT 154594 A	15-02-1997
		AU 3257495 A	07-03-1996
		EP 0775198 A	28-05-1997
		US 5932426 A	03-08-1999

CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C12N 15/37, C07K 14/205, A61K 39/12 — A3

(11) International Publication Number: WO 00/09699

(43) International Publication Date: 24 February 2000 (24.02.00)

(21) International Application Number: PCT/US99/18016

(22) International Filing Date: 13 August 1999 (13.08.99)

(30) Priority Data:
60/096,625 14 August 1998 (14.08.98) US

(71) Applicant *(for all designated States except US)*: CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: BUONAMASSI, Daniela, Tornese [IT/IT]; Chiron SpA, Via Fiorentina 1, I–53100 Siena (IT). GREER, Catherine, E. [US/US]; Chiron Corporation, 4560 Horton Street, Emeryville, CA 94608 (US). GALEOTTI, Cesira, L. [IT/IT]; Chiron SpA, Via Fiorentina 1, I–53100 Siena (IT). BENSI, Giuliano [IT/IT]; Chiron SpA, Via Fiorentina 1, I–53100 Siena (IT). PETRACCA, Roberto [IT/IT]; Chiron SpA, Via Fiorentina 1, I–53100 Siena (IT).

(74) Agent: HARBIN, Alisa, A.; Chiron Corporation, Intellectual Property – R440, P.O. Box 8097, Emeryville, CA 94662–8097 (US).

(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(88) Date of publication of the international search report:
18 May 2000 (18.05.00)

(54) Title: METHOD FOR PRODUCING YEAST EXPRESSED HPV TYPES 6 AND 16 CAPSID PROTEINS

(57) Abstract

Mosaic VLPs of viral capsid proteins from different virus types are described, as are methods of making the same. Specifically, a diploid yeast strain that coexpresses the L1 and L2 capsid proteins of both HPV–6 and HPV–16 as mosaic VLPs is described. The mosaic VLPs induced the production conformational antibodies against both L1 proteins upon administration to mice.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

09/762762

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
369.100		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/18016	13/08/1999	14/08/1998
International Patent Classification (IPC) or national classification and IPC C12N15/37		
Applicant CHIRON CORPORATION et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
13/03/2000	13.12.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Celler, J Telephone No. +49 89 2399 7336

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/18016

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-28 as originally filed

Claims, No.:

1-51 as originally filed

Drawings, sheets:

1/8-8/8 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 37 - 40 (IA).

because:

☒ the said international application, or the said claims Nos. 37 - 40 relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N) Yes: Claims 5, 7 - 9, 12 - 14, 16 - 26, 28 - 40, 42 - 51

	No:	Claims	1 - 4, 6, 10, 11, 15, 27, 41
Inventive step (IS)	Yes:	Claims	5, 7 - 9, 12 - 14, 18 - 26, 28 - 34, 39, 40, 44 - 49
	No:	Claims	1 - 4, 6, 10, 11, 15 - 17, 27, 35 - 38, 41 - 43, 50, 51
Industrial applicability (IA)	Yes:	Claims	1 - 36, 41 - 51
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claims 37 - 40 relate to subject matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject matter of these claims (Article 34(4)(a)(i) PCT).
For the assessment of the present Claims 37 - 40 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims.

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

The present application relates to virus-like particles (VLPs) comprising capsid proteins derived from at least two types of viruses. Said VLPs are produced by co-expression of corresponding genes in diploid cells of Saccharomyces cerevisiae. The methods of expressing the capsid proteins and producing said VLPs are sought to be protected. The generated VLPs are thought to be applicable in production of vaccines for human immunisation, therefore, claims are also directed towards corresponding compositions and methods of treatment.

Reference is made to the fallowing documents:

D1: WO 98 14564 A (LUDMERER STEVEN ;MERCK & CO INC (US)) 9 April 1998 (1998-04-09)

D2: CHANG C ET AL: 'Phenotypic mixing between different hepadnavirus nucleocapsid proteins reveals C protein dimerization to be cis preferential' JOURNAL OF VIROLOGY, vol. 68, no. 8, August 1994 (1994-08), pages

5225-5231, XP002130379 AMERICAN SOCIETY FOR MICROBIOLOGY US cited in the application

D3: WO 96 05293 A (UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION ;BARALLE FRANCESCO) 22 February 1996 (1996-02-22)

D1 describes virus-like particles (VLPs) which are formed from expressed major capsid protein late 1 (L1), wherein the L1 protein is so engineered as to contain amino acid sequences derived from Human Papilloma Virus (HPV) type 11 and 16 (e. g. p. 10, lines 3 - 14 and p. 12, EXAMPLE 4). The VLPs are produced in SF9 cells and during that process they become constituents of various compositions (e. g. p. 11, EXAMPLE 3). Thus, the VLPs of D1 have to be seen as "comprising capsid proteins (L1) from at least two types of viruses" (HPV) and consequently, due to this vague formulation, they fall under the scope of the subject matter of Claims 1 - 4, 6 and 10. That, in turn, render the said claims not novel (Art. 33(2) PCT). It should also be noted that D3 discloses similar VLPs which fall under the scope of the subject matter defined in Claims 1, 2 and 10

D2 discloses mixed VLP formation, wherein the capsid proteins are derived from woodchuck hepatitis virus (WHV) and from ground squirrel hepatitis virus (DSHV). Furthermore, the mixed VLPs are purified on a sucrose gradient (p. 5226, right column, "Sucrose gradient fractionation") and during the process become constituents of a composition. It also appears that such purification process would make the VLPs of D2 suitable for use in immunisation. In consequence, the VLPs of D2 fall under the scope of the subject matter of Claims 1, 2, 10 and 11, which renders again the said claims not new (Art. 33(2) PCT).

Furthermore D2 discloses a method of producing the mixed VLPs by cloning the corresponding genes in appropriate cassettes and expressing said cassettes in the same host cell (e. g. p. 5227, right column, "Interactions among mammalian HBV core proteins"). Even if the cassettes comprise promoters and/or terminators that are not the same but different, the methods and the host cell of present Claims 15 (also dependent Claims 16, 17) , 27 (also dependent Claims 35, 36) and 41 (also dependent Claims 42, 43) would not be regarded as inventive,

because there is no evidence provided in the present application for the existence of any special technical effect associated with the use of the same promoters and/or terminators and because the expression of two types of viral capsid proteins in one cell to form VLPs is known from D2 (Art. 33(3) PCT). On the other hand, if the promoters and/or terminators are the same, the subject matter of Claims 15, 27 and 41 would not be regarded as new (Art. 33(2) PCT). Therefore, at present, novelty of Claims 15, 27 and 41 cannot be acknowledged.
It should also be noted that due to the lack of novelty of Claims 1 - 4, 6, 10 and 11, the dependent Claims 37, 38, 50 and 51, would not be regarded as inventive (Art. 33(3) PCT).

As none of the identified documents of the prior art discloses VLPs of HPV type 6 and 16 and/or said VLPs comprising the major capsid protein late 1 (L1) together with capsid protein late 2 (L2), the subject matter of Claims 5 and 7 - 9 is regarded as new (Art. 33(2) PCT).

D2 discloses that the formation of mixed VLP comprising different polypeptides form two viral species is possible in case of WHV and GSHV but not in case of DHBV. Therefore, the teaching of D2 is that formation of mixed VLPs could take place, it is not obvious, however, for which and how many types or species of viruses it is possible. Therefore a skilled person faced with the problem of provision of such mixed VLPs derived from different types HPVs would need to exercise inventive skills to arrive at the subject matter which is sought to be protected in Claims 5 and 7 - 9. Consequently, said claims are regarded as inventive (Art. 33(3) PCT).

Furthermore, the mixed VLPs of Claims 5 and 7 - 9 are demonstrated in the present application to induce immune responses against viral proteins of both types of HPVs simultaneously. This may be viewed as an alternative to the solution disclosed in D1 to the problem of provision of VLPs that induce immune responses to more than one type of HPV simultaneously. The VLPs of D1 "comprise capsid proteins from at least two types of viruses" wherein the VLP has a polypeptide chain with a sequence composed of those of both types of HPVs. The VLPs of present Claims 5 and 7 - 9 comprise at least two different polypeptide chains, wherein each of them is characterised by the sequence of

different type of HPV. As the identified prior art does not disclose any method of inducing immune responses against VLPs comprising different polypeptide chains derived from more than one HPV type, the method of Claims 39 and 40 is regarded as new (Art 33(2) PCT). Since the method of Claims 39 and 40 has as the essential feature the new and inventive VLPs of present Claims 5 and 7 - 9, Claims 39 and 40 are also inventive (Art. 33(3) PCT).

As Claims 12 - 14 are dependent on new and inventive Claim 9, they are also regarded as new and inventive (Art. 33(2) and (3) PCT).

As the method of Claims 18 - 26 necessarily results in generation of the new and inventive products of Claims 5 and 7 - 9 or other VLPs comprising different polypeptide chains derived from more than one HPV type (see above), which are also new and inventive, Claims 18 - 26 are new and inventive (Art. 33(2) and (3) PCT), provided that the abbreviations L1 and L2, used in said claims refer to capsid proteins of HPV (see also Re Item VIII).

Similar reasoning applies to Claim 28 - 34, which are directed to a host cell expressing capsid proteins from at least two types of viruses wherein said viruses are different types of HPV and to Claims 44 - 49. Consequently Claim 28 - 34 and Claims 44 - 49 are new and inventive (Art. 33(2) and (3) PCT).

Re Item VII

Certain defects in the international application

According to the requirements of Rule 11.13(l) reference signs not appearing in the description shall not appear in the drawings, and vice versa. This requirement is not met in view of the reference sign in "Brief Description of Drawings" (p. 4 - 6). For example, in the description of Figure 4 reference is made to "empty" or "black" boxes, however, by looking at the corresponding figure, no distinction of the boxes can be made according to the description. Similarly, in the description of Figure 6 reference is made to panels "(a and c)" or "(b and d)", however, in the actual figure such panels have not been indicated.

Re Item VIII

Certain observations on the international application

Claims 11 and 50 do not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The claims attempt to define the subject matter in terms of the result to be achieved which merely amounts to a statement of the underlying problem (Claim 11 - purification for immunisation and Claim 50 - induction of immune responses against both types of viruses). The technical features necessary for achieving this result should be added.

The terms MF59, pBS24.1 and pUC8 employed in Claims 14, 25 and 26, respectively, and appearing to be registered trade marks have no precise meaning as they are not internationally accepted as standard descriptive terms, thereby rendering the definition of the subject matter of these claims unclear (Article 6 PCT).

The use of abbreviations such as HPV, VLP, L1 and L2 renders claims unclear, if the abbreviations have not been defined within a block of claims consisting of an independent, and the upon-it-dependent claims. Undefined abbreviations render the scope of the subject matter unclear (Art. 6 PCT).

The terms "non-integrative" and "integrative vector/s" appear obscure in the context of Claims 24 - 26, because a skilled person would not be certain to what technical effect they refer and in consequence, render said claims unclear (Art. 6 PCT).

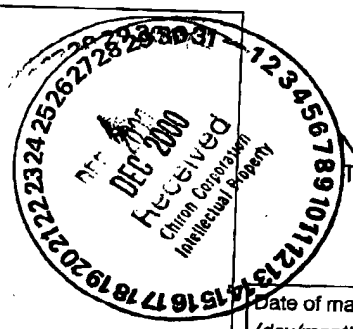
PATENT COOPERATION TREATY

09/762762

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

HARBIN, Alisa A. et al
CHIRON CORPORATION
Intellectual Property - R440
P.O. Box 8097
Emeryville, CA 94662-8097
ETATS-UNIS D'AMERIQUE



PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Applicant's or agent's file reference 369.100	Date of mailing *(day/month/year)* 13.12.2000
	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/18016	13/08/1999	14/08/1998

Applicant
CHIRON CORPORATION et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

12/26/00 UD
A-1H PA
369.100
2/14/01 - NP2

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Vullo, C Tel.+49 89 2399-8061

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY 09/762762

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
369.100		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US99/18016	13/08/1999	14/08/1998
International Patent Classification (IPC) or national classification and IPC		
C12N15/37		
Applicant		
CHIRON CORPORATION et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   I ☒ Basis of the report
   II ☐ Priority
   III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   IV ☐ Lack of unity of invention
   V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   VI ☐ Certain documents cited
   VII ☒ Certain defects in the international application
   VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
13/03/2000	13.12.2000
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Celler, J Telephone No. +49 89 2399 7336

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/18016

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:

Description, pages:

1-28 as originally filed

Claims, No.:

1-51 as originally filed

Drawings, sheets:

1/8-8/8 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/18016

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 37 - 40 (IA).

because:

☒ the said international application, or the said claims Nos. 37 - 40 relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination report cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N) Yes: Claims 5, 7 - 9, 12 - 14, 16 - 26, 28 - 40, 42 - 51

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US99/18016

	No:	Claims	1 - 4, 6, 10, 11, 15, 27, 41
Inventive step (IS)	Yes:	Claims	5, 7 - 9, 12 - 14, 18 - 26, 28 - 34, 39, 40, 44 - 49
	No:	Claims	1 - 4, 6, 10, 11, 15 - 17, 27, 35 - 38, 41 - 43, 50, 51
Industrial applicability (IA)	Yes:	Claims	1 - 36, 41 - 51
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

INTERNATIONAL PRELIMINARY EXAMINATION REPORT - SEPARATE SHEET

International application No. PCT/US99/18016

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claims 37 - 40 relate to subject matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject matter of these claims (Article 34(4)(a)(i) PCT).

For the assessment of the present Claims 37 - 40 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims.

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

The present application relates to virus-like particles (VLPs) comprising capsid proteins derived from at least two types of viruses. Said VLPs are produced by co-expression of corresponding genes in diploid cells of Saccharomyces cerevisiae. The methods of expressing the capsid proteins and producing said VLPs are sought to be protected. The generated VLPs are thought to be applicable in production of vaccines for human immunisation, therefore, claims are also directed towards corresponding compositions and methods of treatment.

Reference is made to the fallowing documents:

D1: WO 98 14564 A (LUDMERER STEVEN ;MERCK & CO INC (US)) 9 April 1998 (1998-04-09)

D2: CHANG C ET AL: 'Phenotypic mixing between different hepadnavirus nucleocapsid proteins reveals C protein dimerization to be cis preferential' JOURNAL OF VIROLOGY, vol. 68, no. 8, August 1994 (1994-08), pages

5225-5231, XP002130379 AMERICAN SOCIETY FOR MICROBIOLOGY US cited in the application

D3: WO 96 05293 A (UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION ;BARALLE FRANCESCO) 22 February 1996 (1996-02-22)

D1 describes virus-like particles (VLPs) which are formed from expressed major capsid protein late 1 (L1), wherein the L1 protein is so engineered as to contain amino acid sequences derived from Human Papilloma Virus (HPV) type 11 and 16 (e. g. p. 10, lines 3 - 14 and p. 12, EXAMPLE 4). The VLPs are produced in SF9 cells and during that process they become constituents of various compositions (e. g. p. 11, EXAMPLE 3). Thus, the VLPs of D1 have to be seen as "comprising capsid proteins (L1) from at least two types of viruses" (HPV) and consequently, due to this vague formulation, they fall under the scope of the subject matter of Claims 1 - 4, 6 and 10. That, in turn, render the said claims not novel (Art. 33(2) PCT). It should also be noted that D3 discloses similar VLPs which fall under the scope of the subject matter defined in Claims 1, 2 and 10

D2 discloses mixed VLP formation, wherein the capsid proteins are derived from woodchuck hepatitis virus (WHV) and from ground squirrel hepatitis virus (DSHV). Furthermore, the mixed VLPs are purified on a sucrose gradient (p. 5226, right column, "Sucrose gradient fractionation") and during the process become constituents of a composition. It also appears that such purification process would make the VLPs of D2 suitable for use in immunisation. In consequence, the VLPs of D2 fall under the scope of the subject matter of Claims 1, 2, 10 and 11, which renders again the said claims not new (Art. 33(2) PCT).

Furthermore D2 discloses a method of producing the mixed VLPs by cloning the corresponding genes in appropriate cassettes and expressing said cassettes in the same host cell (e. g. p. 5227, right column, "Interactions among mammalian HBV core proteins"). Even if the cassettes comprise promoters and/or terminators that are not the same but different, the methods and the host cell of present Claims 15 (also dependent Claims 16, 17) , 27 (also dependent Claims 35, 36) and 41 (also dependent Claims 42, 43) would not be regarded as inventive,

because there is no evidence provided in the present application for the existence of any special technical effect associated with the use of the same promoters and/or terminators and because the expression of two types of viral capsid proteins in one cell to form VLPs is known from D2 (Art. 33(3) PCT). On the other hand, if the promoters and/or terminators are the same, the subject matter of Claims 15, 27 and 41 would not be regarded as new (Art. 33(2) PCT). Therefore, at present, novelty of Claims 15, 27 and 41 cannot be acknowledged.
It should also be noted that due to the lack of novelty of Claims 1 - 4, 6, 10 and 11, the dependent Claims 37, 38, 50 and 51, would not be regarded as inventive (Art. 33(3) PCT).

As none of the identified documents of the prior art discloses VLPs of HPV type 6 and 16 and/or said VLPs comprising the major capsid protein late 1 (L1) together with capsid protein late 2 (L2), the subject matter of Claims 5 and 7 - 9 is regarded as new (Art. 33(2) PCT).

D2 discloses that the formation of mixed VLP comprising different polypeptides form two viral species is possible in case of WHV and GSHV but not in case of DHBV. Therefore, the teaching of D2 is that formation of mixed VLPs could take place, it is not obvious, however, for which and how many types or species of viruses it is possible. Therefore a skilled person faced with the problem of provision of such mixed VLPs derived from different types HPVs would need to exercise inventive skills to arrive at the subject matter which is sought to be protected in Claims 5 and 7 - 9. Consequently, said claims are regarded as inventive (Art. 33(3) PCT).

Furthermore, the mixed VLPs of Claims 5 and 7 - 9 are demonstrated in the present application to induce immune responses against viral proteins of both types of HPVs simultaneously. This may be viewed as an alternative to the solution disclosed in D1 to the problem of provision of VLPs that induce immune responses to more than one type of HPV simultaneously. The VLPs of D1 "comprise capsid proteins from at least two types of viruses" wherein the VLP has a polypeptide chain with a sequence composed of those of both types of HPVs. The VLPs of present Claims 5 and 7 - 9 comprise at least two different polypeptide chains, wherein each of them is characterised by the sequence of

different type of HPV. As the identified prior art does not disclose any method of inducing immune responses against VLPs comprising different polypeptide chains derived from more than one HPV type, the method of Claims 39 and 40 is regarded as new (Art 33(2) PCT). Since the method of Claims 39 and 40 has as the essential feature the new and inventive VLPs of present Claims 5 and 7 - 9, Claims 39 and 40 are also inventive (Art. 33(3) PCT).

As Claims 12 - 14 are dependent on new and inventive Claim 9, they are also regarded as new and inventive (Art. 33(2) and (3) PCT).

As the method of Claims 18 - 26 necessarily results in generation of the new and inventive products of Claims 5 and 7 - 9 or other VLPs comprising different polypeptide chains derived from more than one HPV type (see above), which are also new and inventive, Claims 18 - 26 are new and inventive (Art. 33(2) and (3) PCT), provided that the abbreviations L1 and L2, used in said claims refer to capsid proteins of HPV (see also Re Item VIII).

Similar reasoning applies to Claim 28 - 34, which are directed to a host cell expressing capsid proteins from at least two types of viruses wherein said viruses are different types of HPV and to Claims 44 - 49. Consequently Claim 28 - 34 and Claims 44 - 49 are new and inventive (Art. 33(2) and (3) PCT).

Re Item VII

Certain defects in the international application

According to the requirements of Rule 11.13(l) reference signs not appearing in the description shall not appear in the drawings, and vice versa. This requirement is not met in view of the reference sign in "Brief Description of Drawings" (p. 4 - 6). For example, in the description of Figure 4 reference is made to "empty" or "black" boxes, however, by looking at the corresponding figure, no distinction of the boxes can be made according to the description. Similarly, in the description of Figure 6 reference is made to panels "(a and c)" or "(b and d)", however, in the actual figure such panels have not been indicated.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT - SEPARATE SHEET

International application No. PCT/US99/18016

Re Item VIII

Certain observations on the International application

Claims 11 and 50 do not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The claims attempt to define the subject matter in terms of the result to be achieved which merely amounts to a statement of the underlying problem (Claim 11 - purification for immunisation and Claim 50 - induction of immune responses against both types of viruses). The technical features necessary for achieving this result should be added.

The terms MF59, pBS24.1 and pUC8 employed in Claims 14, 25 and 26, respectively, and appearing to be registered trade marks have no precise meaning as they are not internationally accepted as standard descriptive terms, thereby rendering the definition of the subject matter of these claims unclear (Article 6 PCT).

The use of abbreviations such as HPV, VLP, L1 and L2 renders claims unclear, if the abbreviations have not been defined within a block of claims consisting of an independent, and the upon-it-dependent claims. Undefined abbrevlations render the scope of the subject matter unclear (Art. 6 PCT).

The terms "non-integrative" and "integrative vector/s" appear obscure in the context of Claims 24 - 26, because a skilled person would not be certain to what technical effect they refer and in consequence, render said claims unclear (Art. 6 PCT).

INTERNATIONAL SEARCH REPORT

09/762762

Inter ...nal Application No
PCT/US 99/18016

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/37 C07K14/205 A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 14564 A (LUDMERER STEVEN ;MERCK & CO INC (US)) 9 April 1998 (1998-04-09) examples 5,6	1-4,6,10
X	WO 96 05293 A (UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION ;BARALLE FRANCESCO) 22 February 1996 (1996-02-22) page 13, line 25 -page 17, line 10	1-3

-/—

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 February 2000	28/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Cupido, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/18016

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHANG C ET AL: "Phenotypic mixing between different hepadnavirus nucleocapsid proteins reveals C protein dimerization to be cis preferential" JOURNAL OF VIROLOGY, vol. 68, no. 8, August 1994 (1994-08), pages 5225-5231, XP002130379 AMERICAN SOCIETY FOR MICROBIOLOGY US cited in the application page 5228, left-hand column, last paragraph -page 5229, right-hand column, paragraph 1	1,2
A	HOFMANN K J ET AL: "Sequence conservation within the major capsid protein of human papillomavirus (HPV) type 18 and formation of HPV-18 virus-like particles in Saccharomyces cerevisiae" JOURNAL OF GENERAL VIROLOGY,GB,SOCIETY FOR GENERAL MICROBIOLOGY, READING, vol. 77, no. 3, March 1996 (1996-03), pages 465-468, XP000604634 ISSN: 0022-1317 the whole document	41-51

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Ir.. ..national application No.
PCT/US 99/ 18016

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 37-40 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern[illegible]al Application No
PCT/US 99/18016

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9814564 A	09-04-1998	EP 0956349 A	17-11-1999
		US 5922588 A	13-07-1999
WO 9605293 A	22-02-1996	AT 402898 B	25-09-1997
		AT 154594 A	15-02-1997
		AU 3257495 A	07-03-1996
		EP 0775198 A	28-05-1997
		US 5932426 A	03-08-1999

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PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
C12N 15/37, C07K 14/205, A61K 39/12

A2

(11) International Publication Number: **WO 00/09699**

(43) International Publication Date: 24 February 2000 (24.02.00)

(21) International Application Number: PCT/US99/18016

(22) International Filing Date: 13 August 1999 (13.08.99)

(30) Priority Data:
60/096,625 14 August 1998 (14.08.98) US

(71) Applicant *(for all designated States except US)*: CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).

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(54) Title: METHOD FOR PRODUCING YEAST EXPRESSED HPV TYPES 6 AND 16 CAPSID PROTEINS

(57) Abstract

Mosaic VLPs of viral capsid proteins from different virus types are described, as are methods of making the same. Specifically, a diploid yeast strain that coexpresses the L1 and L2 capsid proteins of both HPV–6 and HPV–16 as mosaic VLPs is described. The mosaic VLPs induced the production conformational antibodies against both L1 proteins upon administration to mice.

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METHOD FOR PRODUCING YEAST EXPRESSED HPV TYPES 6 AND 16 CAPSID PROTEINS

The present application claims priority under 35 U.S.C. § 119(e) to Provisional Application Serial No. 60/096,625, filed August 14, 1998, said application incorporated by reference herein in its entirety.

Field of the Invention

The present invention is related to the production of mosaic virus-like particles comprising capsid proteins of human papilloma virus (HPV) types 6 and 16 capable of inducing immune response against both HPV types.

Background of the Invention

A promising strategy to induce an immune response capable of neutralizing papillomavirus (PV) infections is the use of virus capsid proteins as antigens. In the case of genital human papillomaviruses (HPVs), this approach was hampered by the lack of any *in vivo* or *in vitro* source of sufficient amounts of native virus. In order to overcome this problem, heterologous expression systems have been extensively used to obtain large quantities of capsid proteins and to allow the analysis of their structural and immunological properties. Expression of the major capsid protein late 1 (L1) from different PV types using prokaryotic (25), baculovirus (21, 23, 37, 41, 42, 46), yeast (14, 18, 19, 20, 29) and mammalian expression systems (15, 16, 51), demonstrated that this protein can self-assemble into virus-like particles (VLPs). Coexpression of the minor capsid protein late 2 (L2) is not strictly necessary to obtain VLPs, although its presence increases the efficiency of particle formation (15, 22, 51) and induces anti-L2 neutralizing antibodies (32). The L1 and L2 VLPs appear similar to native virions by electron microscopy (EM). The use of different animal models has shown that VLPs can be very efficient at inducing a protective immune response.

VLPs meet many of the criteria which make them ideal surrogates of native virions. They resemble infectious particles by ultrastructural analysis (16), elicit virus neutralizing antibodies and bind to the putative receptor on the surface of mammalian

cells (28, 31, 33, 44, 47). Most notably, the results obtained with animal models demonstrated that prophylactic immunization with VLPs can be very effective *in vivo*. Cottontail rabbits, calves and dogs immunized with L1 VLPs were protected from subsequent challenge with the homologous PV (20, 23, 41) and passive transfer of immune sera conferred protection to naive animals (20, 41), indicating that an antibody-mediated response plays a major role in preventing virus infection.

Studies with infectious HPV virions, as well as VLPs of different HPV types, strongly suggested, however, that the immune response is predominantly type-specific. Further, the efficacy of VLP-based anti-HPV vaccine candidates cannot be evaluated in animals since these viruses exhibit a high degree of species specificity. Antibody-mediated virus neutralization has been therefore studied using either *in vitro* assays (35, 40) or xenograft systems which allow propagation of infectious virus of specific HPV types (1, 2, 5, 6, 24). The primary conclusion which could be drawn from these experiments was that immunization with HPV VLPs evokes a neutralizing immune response which is predominantly type-specific (6, 7, 34, 35, 36, 48).

Cross-neutralization has been reported between HPV-6 and HPV-11 (92% amino acid sequence identity) (8) and between HPV-16 and HPV-33 (80% amino acid sequence identity) (48). This may indicate the existence of some correlation between protein sequences and structural similarities that could possibly be relevant for the mechanism of capsid assembly. On the basis of these considerations, however, the concept that HPV-6 and HPV-16 L1 proteins may coassemble is not obvious, since the two viruses belong to phylogenetically more distant groups (3, 45) and exhibit a lower (67%) L1 amino acid sequence identity.

Further, while envelope proteins of viruses belonging to very different families can be incorporated into the same envelope (50), nucleocapsid protein mixing appears to be much more restricted. Mixed core particles between Moloney murine leukaemia virus (MuLV) and human immunodeficiency virus (HIV) have been obtained but only when artificial chimeric Gag precursors, containing both HIV and MuLV determinants are coexpressed with wild-type MuLV Gag proteins (10). By using a yeast two-hybrid system based on GAL4-Gag fusion protein expression plasmids, Franke et al. were able to show that the ability of two heterologous Gag

proteins to multimerize was correlated with the genetic relatedness between them (13).

Mixed capsid formation between wild-type Gag proteins has not been reported so far. In the case of the hepadnavirus core (C) protein, Chang et al. (4) have shown that an epitope-tagged truncated hepatitis B virus (HBV) C polypeptide could coassemble in *Xenopus* oocytes with woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV) C proteins but not with that of duck hepatitis B virus (DHBV). This result was not unexpected since the two core protein sequences have diverged significantly and do not show immunological cross-reactivity. When coassembly of C polypeptides of HBV, WHV and GSHV occurred, formation of mixed capsids resulted from the aggregation of different species of homodimers (4).

Several reports have discussed the importance of disulfide bonds for the integrity of native bovine papillomavirus type 1 (BPV-1) virions (26) and VLP structures (25, 38, 39). Li et al. (26) have also shown that the cysteine 424 mutant (C424) of HPV-11 L1 in the carboxy-terminal domain that has been identified as critical for capsid formation (25), is still able to form capsomeres but not VLPs, indicating that this residue may be involved in interpentamer bonding. The essential role of disulfide bonds has been confirmed by a single point mutation of either C176 or C427 in HPV-33 L1 (C428 in HPV-18 L1), which converts all VLP trimers into monomers, allowing capsomere formation but not VLP assembly (39).

It has been recently proved that, by using an *in vitro* infection system and a sensitive reverse transcriptase PCR-based assay (RT-PCR), antisera to HPV-6 VLPs are not able to neutralize authentic HPV-16 virions (48). Since cysteine residues corresponding to those described as involved in disulfide bonding above are conserved in the HPV-6 and HPV-16 L1 proteins, we hypothesized that mosaic VLPs could either result from intra-capsomeric or inter-capsomeric association of the two proteins and/or from interaction between type-specific subsets of capsomeres.

Summary of the Invention

In one aspect, the present invention relates to a method for producing mosaic virus like particles comprising the capsid proteins from at least two types of viruses,

preferably animal, more preferably HPV. In a preferred aspect, the capsid proteins are from HPV types 6 and 16. In a futher preferred aspect, the capsid proteins are L1 and L2 from HPV types 6 and 16.

In a further aspect, the present invention relates to vectors and hosts for expressing the capsid proteins of at least two types of viruses, preferably animal, more preferably HPV. In a preferred aspect, the capsid proteins are from HPV types 6 and 16. In a futher preferred aspect, the capsid proteins are L1 and L2 from HPV types 6 and 16. In a further preferred aspect, the present invention relates to a diploid yeast strain that coexpresses the L1 and L2 capsid proteins of HPV-6 and HPV-16 as mosaic VLPs.

In another aspect, the present invention relates to a method for inducing an immune response against more than one type of virus using mosaic VLPs comprising capsid proteins from each virus type. In a preferred aspect, the mosaic VLPs comprise capsid proteins from animal viruses, more preferably HPV , most preferably HPV types 6 and 16. In a futher preferred aspect, the mosaic VLPs comprise the L1 and L2 capsid proteins from HPV types 6 and 16.

In still another aspect, the present invention relates to an immunogenic virus like particle comprising capsid proteins from different types of viruses, preferably animal, more preferably HPV, most preferably HPV types 6 and 16. In a futher preferred aspect, the mosaic VLPs comprise the L1 and L2 capsid proteins from HPV types 6 and 16.

Brief Description of the Drawings

FIG. 1 is a schematic of the construction of the pBS-6L1 plasmid.

FIG. 2 depicts the recombinant PCR performed in constructing the pBS-6L1 plasmid.

FIG. 3 depicts a Western blot analysis of cell extracts from yeast strains expressing HPV-6 and HPV-16 capsid proteins. Equivalent amounts of total cell extracts from the parental JSC310 strain (lanes 1) and different recombinant strains (lanes 2 and 3) were separated by 10% SDS-PAGE, electrotransferred to nitrocellulose filters and incubated with the H6.C6 (a) or the H16.H5 (c) type-

specific anti-L1 Mabs, and with HPV-6L2 (b) or HPV-16L2 (d) antisera. Lanes 2a and 2c: JSC310-6L1epi; lanes 3a and 3c: JSC310-16L1epi; lanes 2b and 2d: JSC310-6L2epi; lanes 3b and 3d: JSC310-16L2epi. Molecular mass standards (in kDa) are indicated. This multipanel figure and those which follow have been assembled by using Photoshop 4.0 and FreeHand 7.0 programs for Macintosh.

FIG. 4 is a schematic representation of the yeast integrative plasmids YIpAde (a) and YIpLys-L2 (b) vectors. The continuous lines represent pUC vector sequences. The empty box in (a) represents the adenine 2 gene sequence. The black boxes in (b) represent lysine 2 gene fragments, the grey box represents the L2 gene, the empty boxes represent the ADH2/GAP hybrid promoter and the MFα gene transcriptional termination sequence. The arrow in the L2 box indicates the 5'-3' orientation of the coding sequence. Relevant restriction sites are indicated.

FIG. 5 depicts a Western blot analysis of cellular extracts from recombinant haploid and diploid yeast strains. Total cell extracts were separated by 10% SDS-PAGE, electrotransferred to nitrocellulose filters and incubated with anti-HPV-6 L1 (a) and anti-HPV-16 L1 (c) Mabs and with HPV-6 L2 (b) and HPV-16 L2 (d) antisera. Lanes 1: AB110-6L1/16L2; lanes 2: JSC310-16L1/6L2; lanes 3: AB/JS-4L; lanes 4: JSC310-6L2epi; lanes 5: JSC310-16L2epi. Arrows in (b) and (d) indicate the bands corresponding to the L2 proteins. Molecular mass standards (in kDa) are indicated.

FIG. 6 depicts an analysis of fractions from CsCl gradient sedimentation of AB/JS-4L cell extract. (A) Aliquots from fractions 1 to 9 were blotted onto nitrocellulose filters using either (a and c) denaturing and reducing (D) or (b and d) nondenaturing and nonreducing (N) conditions. The filters were incubated with the type-specific anti-L1 H6.C6 (a) and H16.H5 (c) Mabs, and with the conformationally dependent type-specific anti-L1 H6.B10.5 (b) and H16.V5 (d) Mabs. As a control, the anti-HPV-6 and HPV-16 L1 conformational Mabs were incubated with CsCl purified VLPs (e) blotted under either denaturing or nondenaturing conditions. The arrows in A indicate fraction no. 5. (B) Aliquots of fraction no. 5 were subjected to SDS-PAGE, electroblotted on nitrocellulose filters and incubated either with HPV-6 L2 (lane 3a) or HPV-16 L2 (lane 3b) antiserum. As a control, total cell extracts from the

JSC310-6L2epi (lanes 1) and JSC310-16L2epi (lanes 2) strains were used. Molecular mass standards (in kDa) are indicated. Arrows indicate bands corresponding to the L2 proteins.

FIG. 7 depicts an electron microscope (EM) analysis of CsCl purified VLPs. HPV-6 (a), HPV-16 (b) and HPV-6/16 VLPs were adsorbed onto Formvar-carbon coated grids, stained with 4% uranyl acetate and examined under a Zeiss EM10C microscope at a magnification of x 100,000 (Bar=100nm).

FIG. 8 depicts a Western blot analysis of immunoprecipitated VLPs. CsCl banded VLPs from the AB/JS-4L diploid strain were immunoprecipitated with the anti-HPV-6 L1 conformationally dependent H6.B10.5 Mab. The immunoprecipitated proteins were separated using a 15 centimeter (cm) long 10% polyacrylamide SDS-gel, electroblotted on nitrocellulose membrane and incubated either with the anti-HPV-6 L1 specific H6.C6 Mab (a) or with the anti-HPV-16 L1 specific H16.H5 Mab (b). Control reactions, including either VLPs or the conformational Mab only, were set up and processed under identical experimental conditions. Lane 1: VLPs incubated overnight without the Mab; lane 2: Mab incubated overnight; lane 3: VLPs incubated overnight with the H6.B10.5 conformational Mab; lane 4: total cell extract from the JSC310-6L1epi strain; lane 5: total cell extract from the JSC310-16L1epi strain. Arrows indicate a conformational Mab-derived band (A), the L1 bands (B) and a protein A Sepharose-derived band (C).

FIG. 9 depicts a characterization of sera derived from mice immunized with HPV-6, HPV-16 and mosaic VLPs. (A) Comparable amounts of HPV-6 (lanes 1), HPV-16 (lanes 2) and mosaic VLPs (lanes 3) were separated on SDS-PAGE and immunoblotted with antisera from mice immunized with HPV-6 VLPs (a) HPV-16 VLPs (b) and mosaic VLPs (c). (B) Comparable amounts of HPV-6 and HPV-16 VLPs were dot-blotted under denaturing and reducing (D) and nondenaturing and nonreducing (N) conditions and incubated with the S16 antiserum of a mice immunized with mosaic VLPs.

Detailed Description of the Invention

To test the possibility of inducing antibodies against multiple HPV types, we have generated a recombinant yeast diploid strain that coexpresses the HPV-6 and HPV-16 L1 and L2 genes. HPV-6/16 mosaic VLPs were purified from the cell lysate and used as antigens to immunize mice. The data presented below supports the formation of mosaic VLPs comprising all four proteins. The immunoprecipitation experiment strongly suggests that the CsCl purified VLPs represent the result of a reciprocal interaction of the two L1 proteins, rather than the simple coexistence of different VLP types. The fact that the L2 proteins are present in the same CsCl fractions favors the hypothesis that they are incorporated into the VLPs as well, since the L2 protein alone does not band in a CsCl gradient at the same density as L1 VLPs (22). Further, antisera able to recognize conformational epitopes of both L1 proteins were obtained. Although it remains to be confirmed that the immune response elicited by HPV-6/16 VLPs can neutralize the two viruses, the data herein supports using mosaic VLPs to immunize against a broader spectrum of virus types.

A yeast expression system as herein disclosed is preferred. Different laboratories have observed that a *Saccharomyces cerevisiae* expression system can be successfully used to easily purify PV VLPs (14, 18) which are highly efficient at inducing a protective immune response in animal models (20). Yeast-expressed VLPs are able to elicite a specific immune response not only at systemic but also at mucosal level. Lowe et al. have reported the generation of IgG neutralizing antibodies in the sera and genital secretions of African green monkeys immunized intramuscularly with HPV-11 VLPs, adsorbed to aluminum adjuvant (27). Greer et al. have observed the induction of anti-L1 specific IgG and IgA antibodies in the sera and genital secretions of mice immunized intranasally with HPV-6 VLPs, adjuvanted either with *E. coli* heat-labile enterotoxin (LT) or with a LT-derived non toxic mutant (14). Further, yeast expression affords the potential to scale-up to thousands of liters at relatively low cost and many yeast-derived products for human use are already market approved due to their safety.

To express the HPV-6 and HPV-16 L1 and L2 genes in the same yeast cell, we generated a *S. cerevisiae* diploid strain by mating two haploid strains, each

expressing two of the four capsid proteins. In order to obtain expression of the heterologous genes under identical culture conditions, each of them was cloned into the same expression cassette based on the ADH2/GAP glucose-repressible hybrid promoter and the $T_{MF\alpha}$ transcriptional termination sequence. The HPV-6 and HPV-16 L1 proteins were expressed by means of the episomal expression vector pBS24.1. Expression of the HPV-6 and HPV-16 L2 proteins was instead obtained by cloning the expression cassette into an integrative plasmid suitable for insertion into the *lys2* locus of the haploid strain genome (Fig.4b). As a consequence of this cloning strategy, the L1 and L2 gene copy numbers in the haploid strains were different and this resulted in higher expression levels of the L1 proteins. This should resemble the ratio of L1 to L2 observed in native HPV virions, which has been estimated over a range from 5:1 to 30:1 (25). Table 1 lists the parental yeast strains used, the two recombinant haploid strains obtained and the diploid strain resulting from the mating.

TABLE 1. List of parental and recombinant yeast strains with genotypes and HPV expressed genes

Yeast strain	Genotype	Episomal HPV gene	Integrated HPVgene
JSC310	*MAT*a *leu*2-3 *ura*3-52 *prb*1-1122 *pep*4-3 *prc*1-407 *adr*1::DM15 *cir°*		
AB110	*MAT*α *leu*2-3-112 *ura*3-52 *pep*4-3 *his*4-580 *cir°*		
JSC310-6L1epi	*MAT*a *prb*1-1122 *pep*4-3 *prc*1-407 *adr*1::DM15 *cir°*	6L1	
JSC310-16L1epi	*MAT*a *prb*1-1122 *pep*4-3 *prc*1-407 *adr*1::DM15 *cir°*	16L1	
JSC310-6L2epi	*MAT*a *prb*1-1122 *pep*4-3 *prc*1-407 *adr*1::DM15 *cir°*	6L2	
JSC310-16L2epi	*MAT*a *prb*1-1122 *pep*4-3 *prc*1-407 *adr*1::DM15 *cir °*	16L2	
JSC310-6L2int	*MAT*a *leu*2-3 *ura*3-52 *prb*1-1122 *lys*2 *pep*4-3 *prc*1-407 *adr*1::DM15 *cir°*		
6L2			
AB110-16L2int	*MAT*α *leu*2-3-112 *ura*3-52 *pep*4-3 *lys*2 *his*4-580 *cir°*		16L2
JSC310-16L1/6L2	*MAT*a *prb*1-1122 *lys*2 *prc*1-407 *pep*4-3 *ade*2 *adr*1::DM15 *cir°*	16L1	
6L2			
AB110-6L1/16L2	*MAT*α *pep*4-3 *lys*2 *his*4-580 *cir°*	6L1	16L2
AB/JSC-4L	*MAT*a/*MAT*α *PRB*1/*prb*1-1122 *lys*2/*lys*2 *PRC*1/*prc*1-407 *pep*4-3/*pep*4-3	6L1-16L1	6L2-
16L2			
	*HIS*4/*his*4-580 *ADR*1/*adr*1::DM15 *cir°*		

As used herein, the term "mosaic VLP" refers to a VLP comprising capsid proteins from more than one type of virus. VLPs which result from intra- and/or inter-capsomeric association of the proteins are included.

As used herein, the term "type" in reference to viruses includes viruses (animal and plant) within the same family, group, or genus as well as viruses in different families, groups, or genuses.

As used herein, the term "non-integrative" in reference to a vector indicates that the vector does not integrate into the host DNA.

Yeast strains. The *Saccharomyces cerevisiae* haploid strains used were JSC310 (MATa, *leu*2-3, *ura*3-52, *prb*1-1122, *pep*4-3, *prc*1-407, *adr*1::DM15, *cir °*) (17) and AB110 (MATα, *leu*2-3-112, *ura*3-52, *pep*4-3, *his*4-580, *cir °*) (43), provided by Vicky Hines (Chiron Corporation, Emeryville, CA, USA).

Monoclonal and polyclonal antibodies. The H6.C6 and H16.H5 monoclonal antibodies (Mabs), which bind to denatured HPV-6 and HPV-16 L1 proteins, respectively, in addition to the H6.B10.5 and H16.V5 Mabs, specific for HPV-6 and HPV-16 intact VLPs, have been reported by Christensen et al. (8, 9). For Western blot analysis, these Mabs were used at 1:3000 dilution with a 4°C overnight incubation. HPV-16 L2 rabbit antiserum was a gift of Lutz Gissmann (DKFZ, Heidelberg, Germany), while HPV-6 L2 rabbit antisera were kindly provided by Denise Galloway (Fred Hutchinson Cancer Research Center Seattle, Washington) and Robert C. Rose (University of Rochester, NY). All the antisera were used at 1:3000-5000 dilution with a 4°C overnight incubation. Anti-rabbit and anti-mouse peroxidase-conjugated antibodies were from Biosource International (Camarillo, CA) and were used at 1:5000 dilution at room temperature for 1.5 hours.

Example 1

HPV type-specific detection of capsid proteins expressed in yeast.

A single yeast strain which could express the four HPV-6 and HPV-16 L1 and L2 capsid proteins was prepared. A necessary tool in achieving this was the availability of antibodies which reacted specifically or preferentially with the L1 or the L2 protein of only one HPV type. The HPV-6 and HPV-16 L1 and L2 genes were cloned in the episomal vector pBS24.1 (see Example 2 below) and expressed in the *S. cerevisiae* strain JSC310 to test the type specificity of the available antibodies. Fig. 3 shows the results of a Western blot analysis of total cell extracts prepared from the recombinant strains incubated with specific anti-HPV-6 (a) or HPV-16 (c) L1 Mabs and with HPV-6 (b) or HPV-16 (d) L2 antisera. In all cases HPV type-specific bands were detected, although a weak cross-reactivity could be seen for both the L2 antisera. While the HPV-6 and HPV-16 L1 Mabs identified proteins with the expected molecular weight of about 55 kilodalton (kDa), the L2 proteins, as previously reported (11, 12), showed an electrophoretic mobility corresponding to approximately 72-75 kDa, instead of the 55 kDa predicted on the basis of their amino acid sequences.

Example 2

Construction of recombinant plasmids

DNA fragments encoding the HPV proteins were obtained from available recombinant plasmids, either by restriction enzyme digestion or by PCR amplification (Expand High Fidelity PCR System, Boehringer Mannheim), and they were completely sequenced using an Applied Biosystem (Norvalk, CELLTECH, USA) model 373 DNA sequencer.

The episomal yeast expression vector pBS24.1, a yeast "shuttle" vector (17 and Philip J. Barr, Chiron Corporation, Emeryville, CA, USA), containing the leucine 2 (Leu2) and uracil 3 (Ura3) selectable genes was used. In this instance, it was obtained by digesting an available pBS24.1αt6E7 plasmid with Bam HI and Sal I . The pBS24.1αt6E7 plasmid was prepared for the yeast expression of the HPV-6E7 antigen in a secreted form.

The pBS-6L1 plasmid, expressing the HPV-6 L1 protein under the control of the alcohol-dehydrogenase-2-glyceraldehyde-3-phosphate-dehydrogenase (ADH2/GAP) glucose repressible promoter (J. Shuster, Chiron Corporation, Emeryville, CA, USA) and the mating type alpha factor gene transcriptional termination sequence ($T_{MF\alpha}$) was derived from the pBS24.1 plasmid as follows.

The plasmid pBS-6L1 is a yeast expression vector which contains the HPV-6L1 under the control of the ADH2\GAP promoter cloned into BAM HI and Sa1 I sites of the vector pBS24.1. The vector pBS24.1 contains the α-factor terminator, therefore an "expression cassette" for HPV-6 L1 is obtained. The "expression cassette" for HPV-6L1 consists of the following sequences fused together (from 5' to 3'): ADH2\GAP hybrid promoter, HPV-6L1 gene, and α-factor terminator. At the end of the cloning procedures the above "expression cassette" was obtained into the pBS24.1 (17). The vector pBS24.1 may be replicated both in *Escherichia coli* and in *Saccharomyces cerevisiae* since it contains PBR322 sequences (including the origin of replication and the ampicillin resistance gene) and the complete 2μ sequences (including the origin of replication). It also contains the yeast URA3 gene and the yeast LEU2 gene.

A summary of the construction of plasmid pBS24.1-A/G-6L1 is presented schematically in Figure 1. Due to the lack of suitable restriction sites, the fusion between the glucose repressible ADH2\GAP promoter and the L1 ORF has been obtained by means of recombinant PCR. The 1-563 bp segment of the hybrid promoter (1113 bp long) is derived from GAGαt6E7 plasmid whilst the 564-1113 bp are derived from PCR amplification of Gga plasmid (see below). The 1-115 bp segment of L1 sequence (1503 bp long) is derived from PCR amplification of the pAcC13-6L1 plasmid (Greer et al., *J. Clin. Microbiology*, 2058-2063, 1995 and Munemitsu et al., *Mol. Cell. Biol.*, 10:5977-5982, 1990), whilst the 116-1503 bp segment is derived from pAcC13-6L1 plasmid directly. The DNA sequence of HPV 6 is reported in Schwarz et al., *EMBO J.*, 2:2341-2348, 1983.

The GAGαt6E7 plasmid is a derivative of pGEM-3z (Promega) vector in which the following sequence was constructed (from 5' to 3'): ADH2\GAP promoter, an α-factor derived leader sequence, and the HPV-6E7 coding sequence. The GAGαt6E7 plasmid was digested with Bcl I and Xba I. The DH5α derived plasmid DNA could not be cut with Bcl I because the DH5α cells are dam+, but the Bcl I enzyme is inhibited by overlapping dam methylation; in order to obtain a Bcl I digestible DNA the plasmid was transformed in the dam- JM110 *E. coli* cells (Stratagene). The JM110 derived plasmid was digested with Bcl I and Xba I, the fragment containing the vector and the 5' half of the ADH2\GAP promoter was gel purified and set aside for further ligation.

The pAcC13-6L1 plasmid was digested with Xba I, the insert was gel purified and set aside for ligation. The Xba I insert consisted in the L1 sequence from bp 115 to the end of the sequence, including the stop codon.

The recombinant PCR is schematically represented in Figure 2. The sequences of the primers are listed below.

RP5 5'- ACTGATAGTTTGATCAAAGGGGCAAAACGTAGGGGC-3' SEQ ID NO:1

RP6 5'-GTCGCTAGGCCGCCACATGGTGTTTGTTTATGTGTG-3' SEQ ID NO:2

RP7 5'-AAACACACATAAACAAACACCATGTGGCGGCCTAGC-3' SEQ ID NO:3

RP8 5'-GCAGTCACCACCCTGTACAGGTGTATTAGTACACTG-3' SEQ ID NO:4

A first PCR was performed using the RP5 and RP6 primers and the Gga plasmid DNA as template. The Gga plasmid is a pGEM-3z plasmid derivative obtained in the context of the previous procedures for the HPV-6E7 cloning in yeast and contains the ADH2\GAP promoter. The goal of this first PCR was to obtain the 563-1113 bp portion (3' half) of the ADH2\GAP promoter. The RP5 primer overlapped a Bcl I site. A second PCR was performed using the RP7 and RP8 primers and the pAcC6L1 (Greer et al., 1995) plasmid as template. The goal of this second PCR was to amplify the 5' end of the L1 sequence from the initiation codon to the bp 543. The amplified fragment would contain an Xba I site at position 115. The RP6 and RP7 primers were designed in such a way that the 3' end of the first PCR product would anneal to the 5' end of the second PCR product. A third PCR was performed by mixing the first and second amplimers and the external primers RP5 and RP8. During this PCR a joining between first and second amplimers would happen and also an amplification of the joined product.

The expected 1126 bp product of the third PCR was predicted to consist in the 563-1113 (3' half) sequence of the ADH2\GAP promoter joined to the 1-530 (5' end) sequence of the HPV-6L1 ORF. The final PCR product would have a Bcl I site at the 5' end and an Xba I site in the L1 portion of the sequence at position 115. The third PCR product was digested with Bcl I and Xba I and gel purified. The fragment containing the pGEM-3z vector and the 5' half of the promoter coming from the Bcl I-Xba I digestion of the GAGαt6E7 plasmid was ligated with the Bcl I-Xba I digested recombinant PCR product and to the L1 insert coming from the Xba I digestion of pAcC13-6L1 plasmid.

After transformation into DH5α cells, several transformants were obtained. The miniprep DNAs from 14 transformants were digested using Eco RI. The Eco RI enzyme was chosen because by using this enzyme it has been possible to verify both the expected molecular sizes and the correct orientation of the 6L1 fragment. The

6L1 fragment had identical extremities (such as Xba I), therefore the probability for the fragment to assume an opposite orientation was 50%. By using Eco RI the plasmid DNA of the right clones should give two fragments, 2600 and 2700 bp long. The miniprep DNA of the n°8 clone gave a single band on a first gel but by running the gel much more was possible to resolve the 2600 and 2700 bp fragments. Also using Sph I it was possible to have a further indication that the clone n°8 was good. It was, thus, assumed that the clone n°8 contained the correct pGAG-6L1 plasmid consisting in the pGEM-3z vector containing the HPV-6 L1 sequence under the control of the ADH2\GAP promoter.

The ADH2\GAP-HPV-6L1 insert was excised from pGAG-6L1 plasmid by digesting with Bam HI and Sal I, the insert was gel purified and set aside for further ligation. The promoter-L1 fragment and the pBS24.1 vector were ligated and the product of the reaction was transformed into DH5α cells. The miniprep DNAs from 5 transformants were analyzed by digesting the Bam HI and Sal I and the clones A, B, C, and E were selected as good clones exhibiting the right molecular weight pattern.

A clone was transformed in JSC310 strain of *Saccharomyces cerevisiae* by means of electroporation and the cells were plated on URA- plates. Selected transformants were picked from URA- plates and streaked on LEU- plates. Single colonies from LEU -plates were inoculated in LEU- medium. Four clones grown in LEU- medium were reinoculated in YEPD medium. Cell pellets from the four JSC310-6L1 clones, A, B, C and D were frozen at -20°C after 24 and 48 hours of growth in YEPD medium on purpose to check L1 protein expression. Glycerol batches of the four clones were stored at -80°C.

The 6L1 yeast cell pellets were glass beads extracted, soluble and insoluble extracts were separated by means of centrifugation and prepared for SDS-PAGE analysis. Extracts from a strain not containing the pBS-6L1 plasmid (JSC310 cells transformed with pAB24 vector) were also prepared as a negative control. In Coomassie strained gel and in western immunoblot an induced band exhibiting the expected molecular weight was visible. A comparison of the HPV-6L1 expressed in

the yeast JSC310 strain and the same antigen expressed in insect cells showed that the two antigens have similar molecular weight.

The DNA portion of the L1 gene deriving from recombinant PCR (bp 1-115) has been sequenced using the following primer:

5' TAGTTTTAAAACACCAA 3' SEQ ID NO:12.

The primer annealed at the 3' end of the ADH2\GAP promoter, at position -37from the L1 start codon. The pGAG-6L1 plasmid (pGEM-3z containing the ADH2\GAP promoter fused to the L1 sequence) was used as template. By sequencing it was established that no errors occurred during the recombinant PCR manipulations nor in the cloning steps.

To construct the YIpAde integrative plasmid, a 1,059 bp *Xba*I genomic DNA fragment of the *S. cerevisiae* adenine 2 gene (Ade2) was amplified by using the PCR oligonucleotide primers 5'AdeE (5'-GCGGCGAATTCTAGAACAGTTGGTATATTAG-3' SEQ ID NO:5, inserting an *Eco*RI site) and 3'AdeP (5'GCGGCCTGCAGGGTCTAGACTCTTTTCCATATA-3'SEQ ID NO:6, inserting a *Pst*I site). The amplified DNA fragment was cloned into plasmid pUC8 digested with *Eco*RI and *Pst*I and the *Xba*I sites, included in the amplified DNA fragment, were used to excise the insert for yeast transformation. To obtain the integrative YIpLys-L2 expression plasmids, a 1,318 bp genomic DNA fragment of the *S. cerevisiae* lysine 2 (Lys2) gene was amplified by using the PCR oligonucleotide primers 5'LysE (5'-GCGGAATTCCACTAGTAATTACA-3'SEQ ID NO:7, inserting an *Eco*RI site) and 3'LysH (5'-GATGTAAGCTTCTACTAGTTGA-3'SEQ ID NO:8, inserting a *Hind*III site). The amplified DNA fragment was then inserted into pUC8 (derivatives readily available from commercial sources, e.g., Promega) digested with *Eco*RI and *Hind*III, generating a plasmid named YIpLys. A *Bam*HI DNA fragment from pSI3 vector (Isabel Zaror, Chiron Corporation, Emeryville, CA, USA, pBR322 backbone, ADH2/GAP promoter, SOD protein, and $T_{MF\alpha}$), including the ADH2/GAP promoter, the human superoxide dismutase (SOD) gene and the $T_{MF\alpha}$ transcriptional termination sequence, was cloned into the single *Bgl*II restriction site in the Lys2 gene sequence of YIpLys, obtaining a plasmid named YIpLys-SOD. The YIpLys-6L2 plasmid was derived from YIpLys-SOD

replacing the *Nco*I-*Sal*I DNA fragment encoding the SOD gene with the *Nco*I-*Sal*I DNA fragment from pGEM3z-6L2 (Kent Thudium, Chiron Corporation, Emeryville, CA, USA) encoding the HPV-6b L2 open reading frame (ORF). To construct the YIpLys-16L2 plasmid, the L2 gene was amplified from the cloned HPV-16 genomic DNA (kindly provided in this instance by Dennis J. McCance, University of Rochester, NY) by using the PCR oligonucleotide primers DT-5'L2 (5'-CGACACAAACGTTCTGCAA-3'SEQ ID NO:9) and DT-3'L2 (5'-ATTAGTCGACCTAGGCAGCCAAGAGACATC-3'SEQ ID NO:10), including the translation termination codon and a *Sal*I site. The DNA fragment obtained was digested with *Sal*I and cloned into YIpLys-SOD from which the SOD coding sequence had been removed by digestion with *Nco*I, filling-in with Klenow enzyme and digestion with *Sal*I.

The pBS-6L2 and pBS-16L2 episomal expression plasmids were obtained by replacing a *Sac*I-*Sal*I DNA fragment from pBS-6L1, including part of the ADH2/GAP promoter and the entire HPV-6b L1 ORF, with *Sac*I-*Sal*I DNA fragments, derived from either YIpLys-6L2 or YIpLys-16L2, including the corresponding promoter region and the L2 ORF.

To construct the pBS-16L1 episomal expression plasmid, the L1 gene was amplified from cloned HPV-16 genomic DNA by using the PCR oligonucleotide primers DT-5'L1 (5'-TCTCTTGGCTGCCTAGTGAGGCCA-3' SEQ ID NO:11) and DT-3'L1 (5'-CTAGTAATGTCGACTTACAGCTTACGTTTTTTGCG-3'SEQ ID NO:12), comprising the translational termination codon and a *Sal*I site. The amplified DNA fragment was purified from agarose gel and cloned into blunt-ended pSI3 vector from which the SOD gene had been previously removed by digestion with *Nco*I and *Sal*I restriction enzymes and filling-in with Klenow enzyme. From this intermediate construct, a *Sac*I-*Sal*I DNA fragment, including part of the ADH2/GAP promoter and the HPV-16L1 ORF, was purified and used to replace the corresponding *Sac*I-*Sal*I DNA fragment in pBS-6L1.

Example 3

Generation of recombinant yeast strains

The strains JSC310-6L1epi (14), JSC310-16L1epi, JSC310-6L2epi and JSC310-16L2epi, expressing the four capsid proteins by means of episomal vectors, were obtained by transformation of the parental JSC310 strain with the expression plasmids pBS-6L1 (14), pBS-16L1, pBS-6L2 and pBS-16L2.

The JSC310-6L2int and the AB110-16L2int strains were obtained using the following experimental approach. Competent yeast cells were cotransformed with 5μg of *Eco*RI-*Hind*III digested YIpLys-6L2 or YIpLys-16L2 integrative plasmid and 1μg of pBS24.1 episomal vector to allow the selection of transformants. Different clones were tested for growth onto plates of minimal medium (MM) supplemented with α-adipate to select mutants with an inactivated Lys2 gene (49). Correct integration into the *lys*2 locus was verified by PCR analysis by using pairs of oligonucleotide primers complementary to sequences within the expression cassette and the genomic portion of the Lys2 gene. Among the colonies expressing the L2 protein, one was chosen, cured of the pBS24.1 plasmid and tested for the inability to grow in the absence of uracil and leucine. Introduction of the episomal L1 expressing vectors into these strains was carried out following two different strategies. AB110-16L2int was transformed with the pBS-6L1 expression plasmid and selection of transformants on MM plates without leucine and uracil allowed the isolation of the haploid strain AB110-6L1/16L2. The JSC310-6L2int strain was instead cotransformed with the pBS-16L1 expression vector and with the *Xba*I digested YIpAde integrative plasmid. Transformants grown on selective plates were plated on complete yeast extract-peptone medium (YEP) and allowed to grow at 30°C for 3-4 days until colonies (1-2%) developed a red color due to disruption of the *ade*2 locus (52). One of the clones, which showed correct integration into the *ade*2 locus by PCR and L1 and L2 expression by Western blot analysis, was designated JSC310-16L1/6L2.

Generation of the AB/JSC-4L diploid strain was obtained by mixing cultures, in YEP medium containing 5% glucose, of the two haploid strains, AB110-6L1/16L2 and JSC310-16L1/6L2. Selection of the AB/JSC-4L diploid strain required an

additional genetic marker in the haploid JSC310-6L2int strain. This was obtained inactivating the endogenous Ade2 gene by means of the integration plasmid represented in Fig.4a. Diploid cells were selected onto MM plates lacking histidine and adenine.

Expression of the four proteins in the haploid strains and in the strain resulting from their mating was evaluated by Western blot analysis. Fig.5 shows the results of such experiments demonstrating that both the haploid strains AB110-6L1/16L2 (a and d, lanes 1) and JSC310-16L1/6L2 (b and c, lanes 2) expressed the heterologous genes and that the expression of all four proteins was stably maintained in the resulting AB/JS-4L diploid strain (a, b, c and d, lanes 3).

Example 4

Preparation of VLPs

Parental yeast strains were grown in complete YEP medium. Strains transformed with episomal vectors were first cultured in leucine-deficient MM medium with 4% glucose until they reached midlog phase. Expression of the genes under the control of the ADH2/GAP glucose-repressible promoter was induced by diluting these cultures 1:50 into YEP complete medium and culturing the cells at 30°C for 2-3 days. Total cell extracts were prepared from 3.5 optical densities (OD) of yeast cell cultures grown to approximately OD_{600}=20. Cells were lysed with a 10 minute incubation on ice in 0.24 N NaOH and 0.96% β-mercaptoethanol, followed by trichloroacetic acid (TCA) precipitation, ice cold acetone washing and final suspension of the protein pellet in 100 µl of protein loading buffer. To carry out dot-blot experiments where preservation of L1 conformation was necessary, yeast cells were collected, washed, suspended in phosphate-buffered saline (PBS, pH 7.5) and disrupted by vortexing five times for 1 minute in the presence of glass beads (425-600 µm, Sigma).

Frozen yeast cell pellets were thawed in buffer containing 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, 2 mM $MgCl_2$ and 1 mM EGTA (#E3889, Sigma Chemical Co.) and Complete™Protease Inhibitors (#1-697-498, Boehringer Mannheim). Cells were disrupted by vortexing twice for 10 minutes, with a 5 minute interval on ice, in the

presence of glass beads (0.5 ml beads per ml of cell suspension) using a VWRbrand Multi-tube vortexer (VWR Scientific Product). Cellular debris was removed by a 20 minute centrifugation at 2000 x g. The supernatants were then centrifuged through a 40% (w/w) sucrose cushion (2 hour centrifugation at 100,000 x g). The resulting pellets were suspended in PBS, applied to a pre-formed CsCl gradient (1.17-1.57 g/ml) and centrifuged for 24 hours at 285,000 x g. The gradients were fractionated and aliquots from each fraction were subjected to Western blot analysis with type-specific anti-L1 and anti-L2 antibodies. Peak fractions were pooled and dialyzed against PBS. Total protein concentration was determined by BCA™ Protein Assay Reagent (#23225, Pierce Chemicals).

Example 5

Characterization of VLPs

Proteins were analyzed by denaturing sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10% polyacrylamide) and Western blotting onto nitrocellulose membrane (pore size 0.45 μm, MSI, Westborough, MA USA) according to standard protocols. Dot-blot analysis of denatured and reduced VLPs was carried out boiling the protein samples for 5 minutes in the presence of dithiothreitol (DTT) before applying them to nitrocellulose filters using a bio-dot apparatus (Biorad). When native VLP structure had to be maintained, VLPs in PBS were applied to the membrane without boiling and in the absence of DTT. Reaction with HPV-specific antibodies was detected using the Enhanced Chemiluminescence (ECL) Western blotting reagent (Amersham) and Hyperfilm ECL (Amersham).

Specifically, the cell extract from the diploid strain was subjected to CsCl gradient sedimentation and aliquots of the collected fractions were boiled in the presence of DTT and blotted in duplicate onto nitrocellulose filters. The filters were incubated with anti-HPV-6 and anti-HPV-16 specific Mabs which react with denatured L1 (8, 9), revealing that the two L1 proteins were enriched in the same fractions (Fig. 6A, a and c). The dot-blot experiment was repeated without denaturing and without reducing the protein samples and using anti-HPV-6 and HPV-16 L1 specific Mabs which were previously reported to react exclusively with intact VLPs

in enzyme-linked immunosorbent assay (ELISA) experiments (8, 9). The result obtained confirmed that the two conformationally dependent Mabs were able to recognize the L1 proteins which copurified in the CsCl fractions (Fig 6A, b and d). As expected, the two Mabs reacted specifically with HPV-6 and HPV-16 control VLPs only under nondenaturing and nonreducing conditions (Fig. 6A, e). Western blot analysis of fraction 5 confirmed that both HPV-6 and HPV-16 L2 proteins were also present (Fig. 6B, a and b). Estimation of the refractive index of the identified protein peak gave a value of 1.29-1.3 mg/ml. EM analysis of the enriched fraction revealed the presence of VLPs which appeared to be similar to control VLPs formed by either HPV-6 or HPV-16 L1 (Fig.7).

To evaluate whether the HPV-6 and HPV-16 L1 proteins could interact and assemble into mosaic VLPs, we performed immunoprecipitation experiments using CsCl banded VLPs and the specific anti-HPV-6 L1 conformationally dependent Mab H6.B10.5 (9). Approximately 1 µg of CsCL banded VLPs were diluted with PBS and incubated with the conformationally dependent anti-HPV-6 L1 Mab H6.B10.5 (1:1000 dilution) overnight at 4° C with gentle shaking. The immune complexes were collected with Protein A Sepharose CL-4B (Pharmacia Biotech), washed 4 times with 1 ml PBS, suspended in sample buffer, boiled for 5 minutes, subjected to SDS-PAGE and analyzed by Western blot using anti-HPV-6 and anti-HPV-16 L1 Mabs. The Western blot carried out on the immunoprecipitates using type-specific anti-L1 Mabs (Fig. 8) identified three major bands: (A) was a Mab-derived band, since it could be also observed when the conformational Mab was immunoblotted with the anti-mouse antibody; (B) was a band that appeared only when the VLPs were incubated with the conformational anti-HPV-6 L1 Mab (lanes 3), identifying specifically immunoprecipitated proteins with an electrophoretic mobility corresponding to that of HPV-6 L1 (a, lane 4) and HPV-16 L1 (b, lane 5); (C) was a resin-derived band that was also detected when an aliquot of protein A Sepharose was suspended in PBS and immunoblotted with the anti-mouse antibody. Bands (B) were not visible when the immunoprecipitation was carried out using an unrelated Mab Similarly, HPV-16 L1 could not be detected when HPV-6 and HPV-16 VLPs were mixed and immunoprecipitated.

Example 6

Mouse immunization with VLPs.

To investigate whether HPV-6/16 mosaic VLPs were able to induce an immune response directed against both HPV types, groups of mice were immunized subcutaneously with HPV-6, HPV-16 and mosaic VLPs and the sera were tested after the third immunization. Six week old female Balb/c mice were injected subcutaneously with 20 μg of the following purified antigens: (I) HPV-6 VLPs, (ii) HPV-16 VLPs, (iii) HPV-6/16 VLPs. All the antigens were administered with equal volume of MF59 adjuvant (30). A group of control mice was injected only with MF59. The mice were boosted with 15 μg of the respective antigen at week 3 and 10 μg at week 5. Serum samples were collected on day 12 after the final booster and assayed for capsid protein specific antibodies.

Figure 9A shows the result of the Western blot carried out with the three types of denatured VLPs incubated with three sera, each representative of the different groups of immunized mice. While the reactivity of the sera from mice immunized either with HPV-6 or HPV-16 VLPs was predominantly type-specific (Fig. 9A, a and b), the serum from mouse 16 (S16), immunized with HPV6/16 VLPs, reacted against both HPV-6 and HPV-16 L1 (Fig. 9A, c). To analyze whether the immune response was also directed against conformational epitopes of the L1 proteins, equal amounts of either HPV-6 or HPV-16 VLPs were blotted under denaturing and nondenaturing conditions and incubated with the S16 antiserum. Figure 9B shows that the signal was significantly lower when the samples were denatured and reduced, suggesting that conformational antibodies had been elicited.

The foregoing examples are meant to illustrate the invention and are not to be construed to limit the invention in any way. Those skilled in the art will recognize modifications that are within the spirit and scope of the invention. All references cited herein are hereby incorporated by reference in their entirety.

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What is claimed is:

1. A virus-like particle (VLP) comprising capsid proteins from at least two types of viruses.
2. The VLP of claim 1 wherein said viruses are animal viruses.
3. The VLP of claim 2 wherein said viruses are human viruses.
4. The VLP of claim 3 wherein said viruses are different types of Human Papilloma Virus (HPV).
5. The VLP of claim 4 wherein said types of HPV are types 6 and 16.
6. The VLP of claim 4 or 5 wherein the capsid proteins comprise the major capsid protein late 1 (L1).
7. The VLP of claim 4 or 5 wherein the capsid proteins comprise the minor capsid protein late 2 (L2).
8. The VLP of claim 4 or 5 wherein the capsid proteins comprise L1 from one virus type and L2 from a second virus type.
9. The VLP of claim 6, further comprising the L2 capsid protein.
10. A composition comprising the VLP of claim 1.
11. The composition of claim 10 wherein the VLP is purified for immunization.
12. The composition of claim 11 wherein the VLP comprises the VLP of claim 9.
13. The composition of claim 12, further comprising an adjuvant.
14. The composition of claim 13 wherein the adjuvant is MF59.
15. A method for producing VLPs comprising capsid proteins from at least two types of viruses, said method comprising

a) cloning said capsid proteins into expression cassettes comprising the same promoters and termination sequences; and

b) expressing said cassettes in the same host cell.

16. The method of claim 15 wherein the host cell is a yeast cell.
17. The method of claim 16 wherein the yeast is *Saccharomyces cerevisiae*.
18. The method of claim 15 wherein said viruses are different types of HPV.
19. The method of claim 18 wherein said types of HPV are type 6 and 16.
20. The method of claim 15 or 18 wherein the capsid proteins comprise L1.

21. The method of claim 15 or 18 wherein the capsid proteins comprise L2.

22. The method of claim 15 or 18 wherein the capsid proteins comprise L1 from one virus type and L2 from a second virus type.

23. The method of claim 20, further comprising L2 capsid proteins.

24. The method of claim 23 wherein said L1 protein expression cassettes are cloned into non-integrative vectors, and said L2 proteins expression cassettes are cloned into integrative vectors.

25. The method of claim 24 wherein the non-integrative vector is pBS24.1.

26. The method of claim 24 wherein the integrative vector is pUC8.

27. A host cell comprising vectors for expressing capsid proteins from at least two types of viruses.

28. The host cell of claim 27 wherein said viruses are different types of HPV.

29. The host cell of claim 28 wherein said types of HPV are types 6 and 16.

30. The host cell of claim 29 wherein said capsid proteins comprise L1.

31. The host cell of claim 29 wherein said capsid proteins comprise L2.

32. The host cell of claim 27 or 29 wherein said capsid proteins comprise L1 from one virus type and L2 from a second virus type.

33. The host cell of claim 30, further comprising L2 capsid proteins.

34. The host cell of claim 33 wherein said host cell is a diploid cell.

35. The host cell of claim 27 or 34 wherein said host cell is yeast.

36. The host cell of claim 35 wherein said yeast is *Saccharomyces cerevisiae.*

37. A method for inducing an immune response against more than one type of virus comprising administering the VLP of any of claims 1-5 or 9.

38. A method for inducing an immune response against more than one type of virus comprising administering the VLP of claim 6.

39. A method for inducing an immune response against more than one type of virus comprising administering the VLP of claim 7.

40. A method for inducing an immune response against more than one type of virus comprising administering the VLP of claim 8.

41. A method for expressing capsid proteins from at least two types of viruses, said method comprising

a) cloning said capsid proteins into expression cassettes comprising the same promoters and termination sequences; and

b) expressing said cassettes in the same host cell.

42. The method of claim 41 wherein the host cell is a yeast cell.

43. The method of claim 42 wherein the yeast is *Saccharomyces cerevisiae*.

44. The method of claim 41 wherein said viruses are different types of HPV.

45. The method of claim 44 wherein said types of HPV are type 6 and 16.

46. The method of claim 41 or 45 wherein the capsid proteins comprise L1.

47. The method of claim 41 or 45 wherein the capsid proteins comprise L2.

48. The method of claim 41 or 45 wherein the capsid proteins comprise L1 from one virus type and L2 from a second virus type.

49. The method of claim 46, further comprising L2 capsid proteins.

50. The VLP of claim 1 wherein said VLP induces an immune response against both types of viruses.

51. A composition comprising the VLP of claim 50.

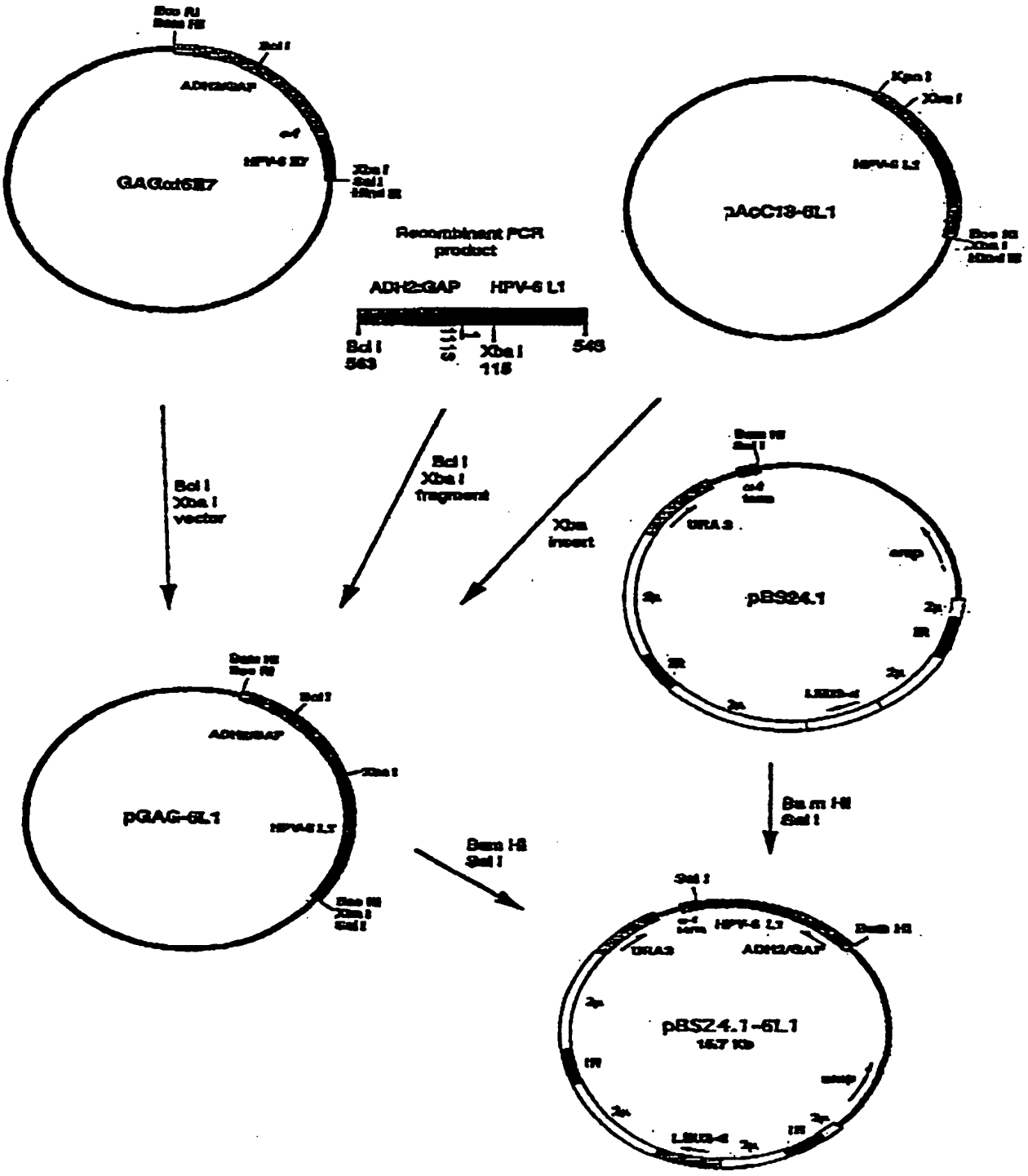

GAGadr6E7
Recombinant PCR product
ADH2/GAP
HPV-6 L1
Bcl I
563
Xba I
116
548
pAcC13-6L1
Bcl I
Xba I
vector
Bcl I
Xba I
fragment
Xba I
insert
pBS24.1
pGAG-6L1
Bam HI
Sal I
pBS24.1-6L1

FIGURE 1

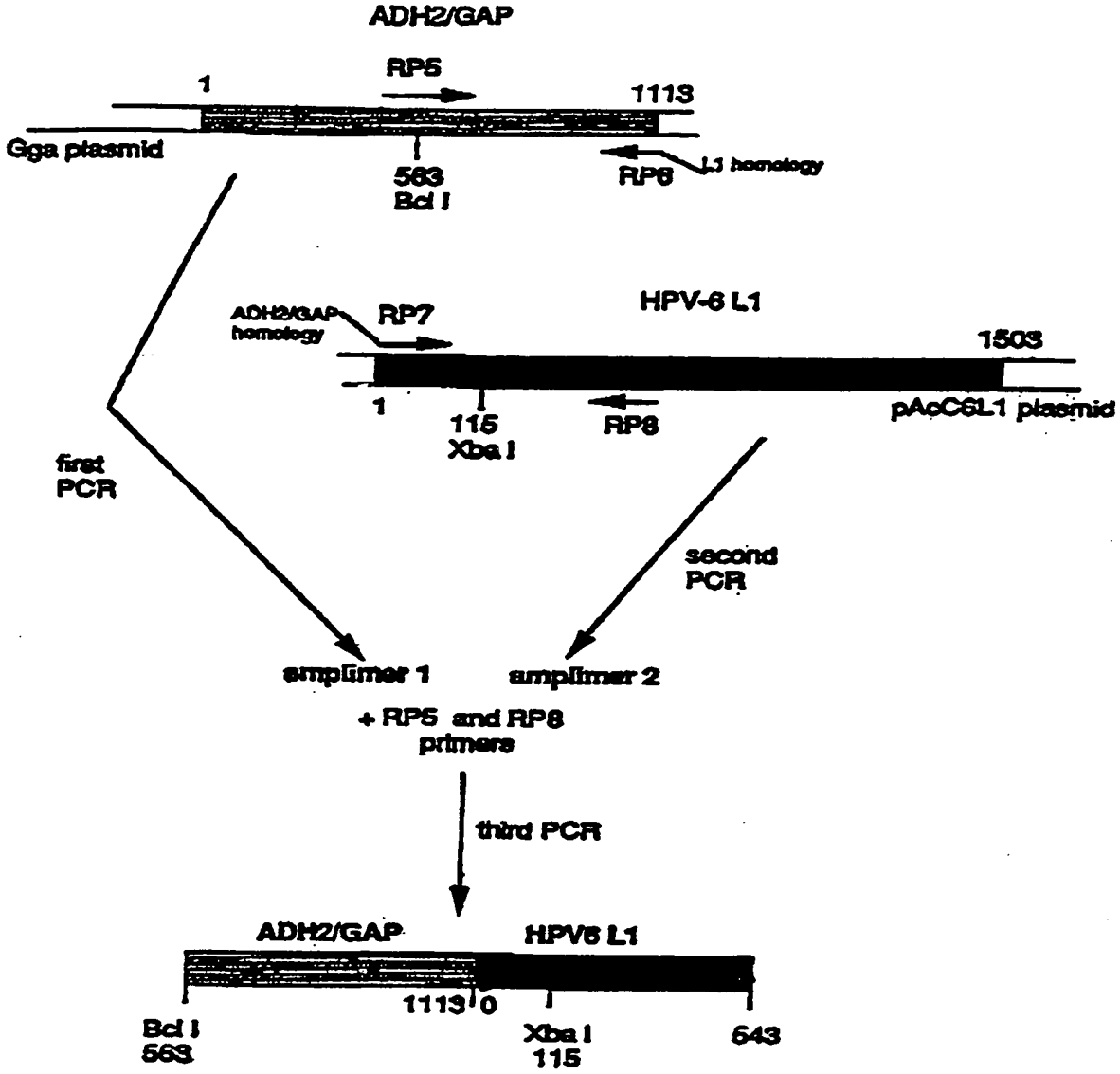

ADH2/GAP
RP5
1
1113
Gga plasmid
563
Bcl I
RP6
L1 homology
HPV-6 L1
ADH2/GAP homology
RP7
1503
1
115
Xba I
RP8
pAcC6L1 plasmid
first PCR
second PCR
amplimer 1
amplimer 2
+ RP5 and RP8 primers
third PCR
ADH2/GAP
HPV6 L1
1113
0
Bcl I
563
Xba I
115
643

FIGURE 2

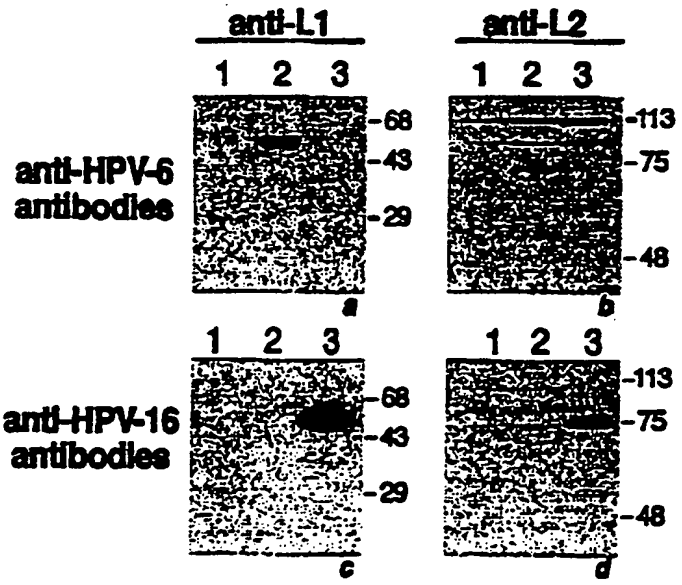

anti-L1
anti-L2
1 2 3
1 2 3
anti-HPV-6 antibodies
anti-HPV-16 antibodies
68
43
29
113
75
48
a
b
c
d

FIGURE 3

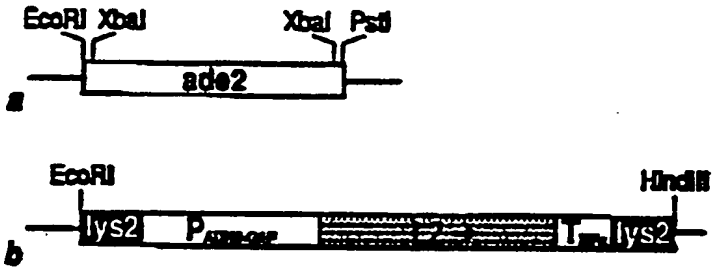
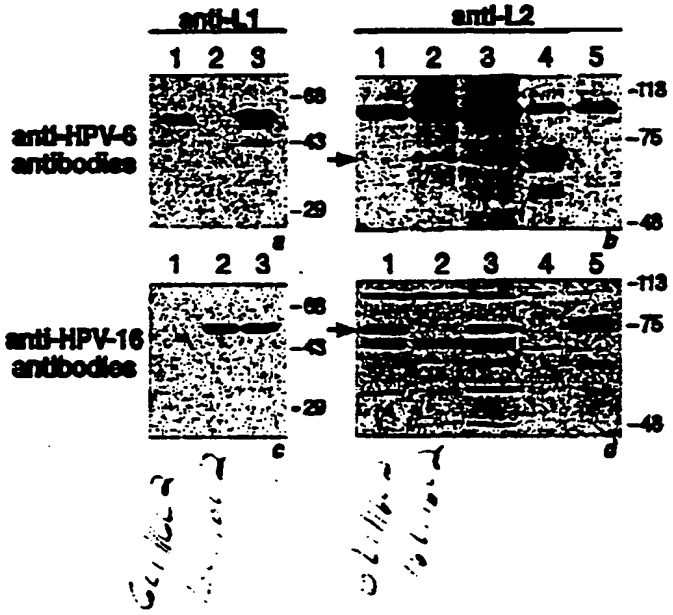


FIGURE 4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anti-L2
1 2 3
1 2 3 4 5
anti-HPV-6 antibodies
anti-HPV-16 antibodies
-66
-43
-29
-118
-75
-48
-113
a
b
c
d

FIGURE 5

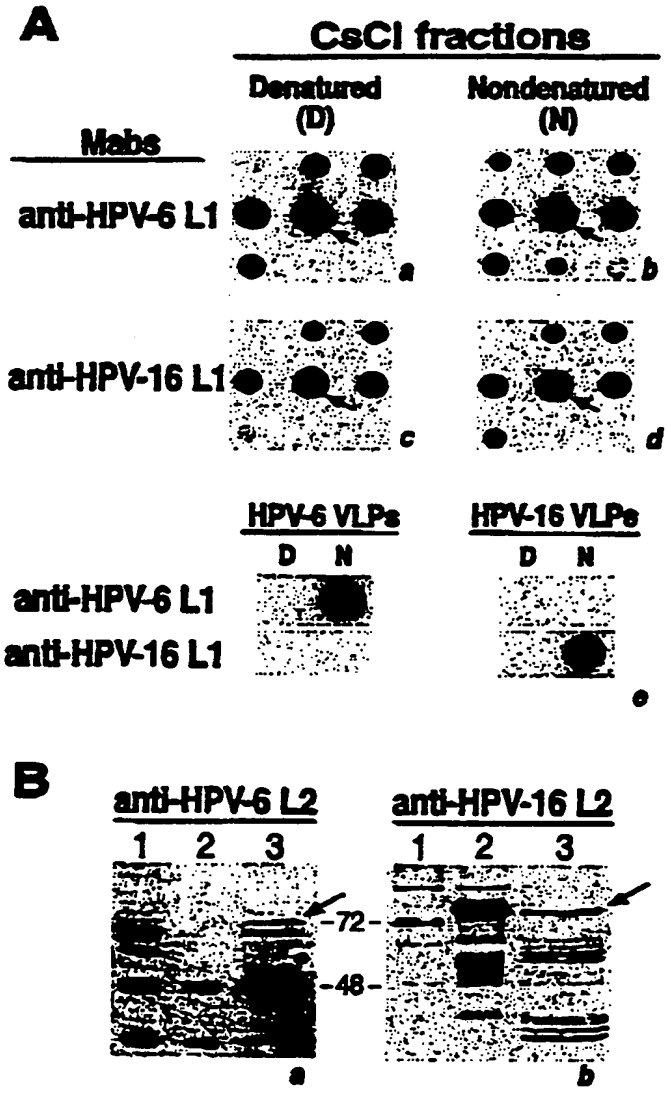
A
CsCl fractions
Denatured (D)
Nondenatured (N)
Mabs
anti-HPV-6 L1
anti-HPV-16 L1
a
b
c
d
HPV-6 VLPs
HPV-16 VLPs
D N
D N
anti-HPV-6 L1
anti-HPV-16 L1
e
B
anti-HPV-6 L2
anti-HPV-16 L2
1 2 3
1 2 3
72
48
a
b

FIGURE 6

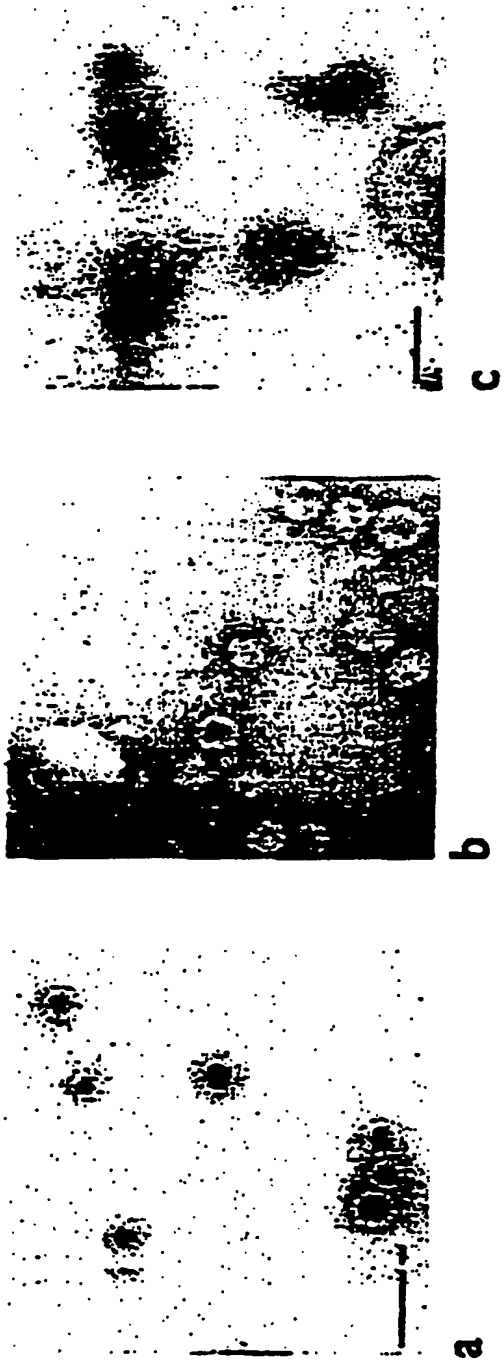

FIGURE 7

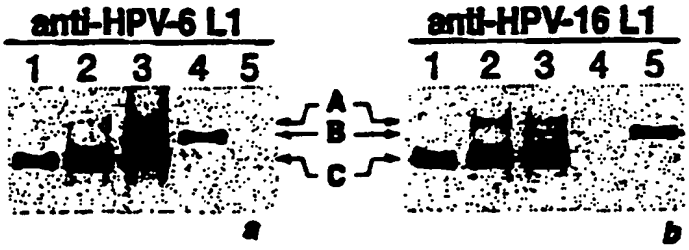

FIGURE 8

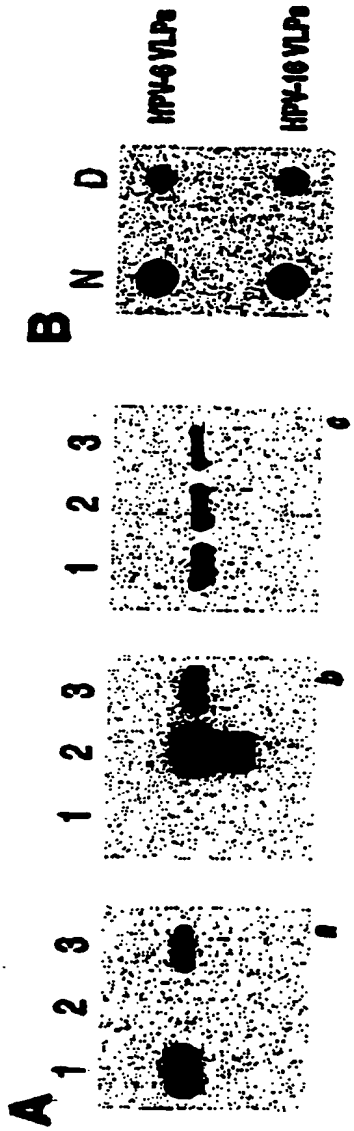

FIGURE 9

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(57) Abstract

Mosaic VLPs of viral capsid proteins from different virus types are described, as are methods of making the same. Specifically, a diploid yeast strain that coexpresses the L1 and L2 capsid proteins of both HPV–6 and HPV–16 as mosaic VLPs is described. The mosaic VLPs induced the production conformational antibodies against both L1 proteins upon administration to mice.

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EE	Estonia	**LR**	Liberia	**SG**	Singapore		

METHOD FOR PRODUCING YEAST EXPRESSED HPV TYPES 6 AND 16 CAPSID PROTEINS

The present application claims priority under 35 U.S.C. § 119(e) to Provisional Application Serial No. 60/096,625, filed August 14, 1998, said application incorporated by reference herein in its entirety.

Field of the Invention

The present invention is related to the production of mosaic virus-like particles comprising capsid proteins of human papilloma virus (HPV) types 6 and 16 capable of inducing immune response against both HPV types.

Background of the Invention

A promising strategy to induce an immune response capable of neutralizing papillomavirus (PV) infections is the use of virus capsid proteins as antigens. In the case of genital human papillomaviruses (HPVs), this approach was hampered by the lack of any *in vivo* or *in vitro* source of sufficient amounts of native virus. In order to overcome this problem, heterologous expression systems have been extensively used to obtain large quantities of capsid proteins and to allow the analysis of their structural and immunological properties. Expression of the major capsid protein late 1 (L1) from different PV types using prokaryotic (25), baculovirus (21, 23, 37, 41, 42, 46), yeast (14, 18, 19, 20, 29) and mammalian expression systems (15, 16, 51), demonstrated that this protein can self-assemble into virus-like particles (VLPs). Coexpression of the minor capsid protein late 2 (L2) is not strictly necessary to obtain VLPs, although its presence increases the efficiency of particle formation (15, 22, 51) and induces anti-L2 neutralizing antibodies (32). The L1 and L2 VLPs appear similar to native virions by electron microscopy (EM). The use of different animal models has shown that VLPs can be very efficient at inducing a protective immune response.

VLPs meet many of the criteria which make them ideal surrogates of native virions. They resemble infectious particles by ultrastructural analysis (16), elicit virus neutralizing antibodies and bind to the putative receptor on the surface of mammalian

cells (28, 31, 33, 44, 47). Most notably, the results obtained with animal models demonstrated that prophylactic immunization with VLPs can be very effective *in vivo*. Cottontail rabbits, calves and dogs immunized with L1 VLPs were protected from subsequent challenge with the homologous PV (20, 23, 41) and passive transfer of immune sera conferred protection to naive animals (20, 41), indicating that an antibody-mediated response plays a major role in preventing virus infection.

Studies with infectious HPV virions, as well as VLPs of different HPV types, strongly suggested, however, that the immune response is predominantly type-specific. Further, the efficacy of VLP-based anti-HPV vaccine candidates cannot be evaluated in animals since these viruses exhibit a high degree of species specificity. Antibody-mediated virus neutralization has been therefore studied using either *in vitro* assays (35, 40) or xenograft systems which allow propagation of infectious virus of specific HPV types (1, 2, 5, 6, 24). The primary conclusion which could be drawn from these experiments was that immunization with HPV VLPs evokes a neutralizing immune response which is predominantly type-specific (6, 7, 34, 35, 36, 48).

Cross-neutralization has been reported between HPV-6 and HPV-11 (92% amino acid sequence identity) (8) and between HPV-16 and HPV-33 (80% amino acid sequence identity) (48). This may indicate the existence of some correlation between protein sequences and structural similarities that could possibly be relevant for the mechanism of capsid assembly. On the basis of these considerations, however, the concept that HPV-6 and HPV-16 L1 proteins may coassemble is not obvious, since the two viruses belong to phylogenetically more distant groups (3, 45) and exhibit a lower (67%) L1 amino acid sequence identity.

Further, while envelope proteins of viruses belonging to very different families can be incorporated into the same envelope (50), nucleocapsid protein mixing appears to be much more restricted. Mixed core particles between Moloney murine leukaemia virus (MuLV) and human immunodeficiency virus (HIV) have been obtained but only when artificial chimeric Gag precursors, containing both HIV and MuLV determinants are coexpressed with wild-type MuLV Gag proteins (10). By using a yeast two-hybrid system based on GAL4-Gag fusion protein expression plasmids, Franke et al. were able to show that the ability of two heterologous Gag

proteins to multimerize was correlated with the genetic relatedness between them (13).

Mixed capsid formation between wild-type Gag proteins has not been reported so far. In the case of the hepadnavirus core (C) protein, Chang et al. (4) have shown that an epitope-tagged truncated hepatitis B virus (HBV) C polypeptide could coassemble in *Xenopus* oocytes with woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV) C proteins but not with that of duck hepatitis B virus (DHBV). This result was not unexpected since the two core protein sequences have diverged significantly and do not show immunological cross-reactivity. When coassembly of C polypeptides of HBV, WHV and GSHV occurred, formation of mixed capsids resulted from the aggregation of different species of homodimers (4).

Several reports have discussed the importance of disulfide bonds for the integrity of native bovine papillomavirus type 1 (BPV-1) virions (26) and VLP structures (25, 38, 39). Li et al. (26) have also shown that the cysteine 424 mutant (C424) of HPV-11 L1 in the carboxy-terminal domain that has been identified as critical for capsid formation (25), is still able to form capsomeres but not VLPs, indicating that this residue may be involved in interpentamer bonding. The essential role of disulfide bonds has been confirmed by a single point mutation of either C176 or C427 in HPV-33 L1 (C428 in HPV-18 L1), which converts all VLP trimers into monomers, allowing capsomere formation but not VLP assembly (39).

It has been recently proved that, by using an *in vitro* infection system and a sensitive reverse transcriptase PCR-based assay (RT-PCR), antisera to HPV-6 VLPs are not able to neutralize authentic HPV-16 virions (48). Since cysteine residues corresponding to those described as involved in disulfide bonding above are conserved in the HPV-6 and HPV-16 L1 proteins, we hypothesized that mosaic VLPs could either result from intra-capsomeric or inter-capsomeric association of the two proteins and/or from interaction between type-specific subsets of capsomeres.

Summary of the Invention

In one aspect, the present invention relates to a method for producing mosaic virus like particles comprising the capsid proteins from at least two types of viruses,

preferably animal, more preferably HPV. In a preferred aspect, the capsid proteins are from HPV types 6 and 16. In a futher preferred aspect, the capsid proteins are L1 and L2 from HPV types 6 and 16.

In a further aspect, the present invention relates to vectors and hosts for expressing the capsid proteins of at least two types of viruses, preferably animal, more preferably HPV. In a preferred aspect, the capsid proteins are from HPV types 6 and 16. In a futher preferred aspect, the capsid proteins are L1 and L2 from HPV types 6 and 16. In a further preferred aspect, the present invention relates to a diploid yeast strain that coexpresses the L1 and L2 capsid proteins of HPV-6 and HPV-16 as mosaic VLPs.

In another aspect, the present invention relates to a method for inducing an immune response against more than one type of virus using mosaic VLPs comprising capsid proteins from each virus type. In a preferred aspect, the mosaic VLPs comprise capsid proteins from animal viruses, more preferably HPV , most preferably HPV types 6 and 16. In a futher preferred aspect, the mosaic VLPs comprise the L1 and L2 capsid proteins from HPV types 6 and 16.

In still another aspect, the present invention relates to an immunogenic virus like particle comprising capsid proteins from different types of viruses, preferably animal, more preferably HPV, most preferably HPV types 6 and 16. In a futher preferred aspect, the mosaic VLPs comprise the L1 and L2 capsid proteins from HPV types 6 and 16.

Brief Description of the Drawings

FIG. 1 is a schematic of the construction of the pBS-6L1 plasmid.

FIG. 2 depicts the recombinant PCR performed in constructing the pBS-6L1 plasmid.

FIG. 3 depicts a Western blot analysis of cell extracts from yeast strains expressing HPV-6 and HPV-16 capsid proteins. Equivalent amounts of total cell extracts from the parental JSC310 strain (lanes 1) and different recombinant strains (lanes 2 and 3) were separated by 10% SDS-PAGE, electrotransferred to nitrocellulose filters and incubated with the H6.C6 (a) or the H16.H5 (c) type-

specific anti-L1 Mabs, and with HPV-6L2 (b) or HPV-16L2 (d) antisera. Lanes 2a and 2c: JSC310-6L1epi; lanes 3a and 3c: JSC310-16L1epi; lanes 2b and 2d: JSC310-6L2epi; lanes 3b and 3d: JSC310-16L2epi. Molecular mass standards (in kDa) are indicated. This multipanel figure and those which follow have been assembled by using Photoshop 4.0 and FreeHand 7.0 programs for Macintosh.

FIG. 4 is a schematic representation of the yeast integrative plasmids YIpAde (a) and YIpLys-L2 (b) vectors. The continuous lines represent pUC vector sequences. The empty box in (a) represents the adenine 2 gene sequence. The black boxes in (b) represent lysine 2 gene fragments, the grey box represents the L2 gene, the empty boxes represent the ADH2/GAP hybrid promoter and the MFα gene transcriptional termination sequence. The arrow in the L2 box indicates the 5'-3' orientation of the coding sequence. Relevant restriction sites are indicated.

FIG. 5 depicts a Western blot analysis of cellular extracts from recombinant haploid and diploid yeast strains. Total cell extracts were separated by 10% SDS-PAGE, electrotransferred to nitrocellulose filters and incubated with anti-HPV-6 L1 (a) and anti-HPV-16 L1 (c) Mabs and with HPV-6 L2 (b) and HPV-16 L2 (d) antisera. Lanes 1: AB110-6L1/16L2; lanes 2: JSC310-16L1/6L2; lanes 3: AB/JS-4L; lanes 4: JSC310-6L2epi; lanes 5: JSC310-16L2epi. Arrows in (b) and (d) indicate the bands corresponding to the L2 proteins. Molecular mass standards (in kDa) are indicated.

FIG. 6 depicts an analysis of fractions from CsCl gradient sedimentation of AB/JS-4L cell extract. (A) Aliquots from fractions 1 to 9 were blotted onto nitrocellulose filters using either (a and c) denaturing and reducing (D) or (b and d) nondenaturing and nonreducing (N) conditions. The filters were incubated with the type-specific anti-L1 H6.C6 (a) and H16.H5 (c) Mabs, and with the conformationally dependent type-specific anti-L1 H6.B10.5 (b) and H16.V5 (d) Mabs. As a control, the anti-HPV-6 and HPV-16 L1 conformational Mabs were incubated with CsCl purified VLPs (e) blotted under either denaturing or nondenaturing conditions. The arrows in A indicate fraction no. 5. (B) Aliquots of fraction no. 5 were subjected to SDS-PAGE, electroblotted on nitrocellulose filters and incubated either with HPV-6 L2 (lane 3a) or HPV-16 L2 (lane 3b) antiserum. As a control, total cell extracts from the

JSC310-6L2epi (lanes 1) and JSC310-16L2epi (lanes 2) strains were used. Molecular mass standards (in kDa) are indicated. Arrows indicate bands corresponding to the L2 proteins.

FIG. 7 depicts an electron microscope (EM) analysis of CsCl purified VLPs. HPV-6 (a), HPV-16 (b) and HPV-6/16 VLPs were adsorbed onto Formvar-carbon coated grids, stained with 4% uranyl acetate and examined under a Zeiss EM10C microscope at a magnification of x 100,000 (Bar=100nm).

FIG. 8 depicts a Western blot analysis of immunoprecipitated VLPs. CsCl banded VLPs from the AB/JS-4L diploid strain were immunoprecipitated with the anti-HPV-6 L1 conformationally dependent H6.B10.5 Mab. The immunoprecipitated proteins were separated using a 15 centimeter (cm) long 10% polyacrylamide SDS-gel, electroblotted on nitrocellulose membrane and incubated either with the anti-HPV-6 L1 specific H6.C6 Mab (a) or with the anti-HPV-16 L1 specific H16.H5 Mab (b). Control reactions, including either VLPs or the conformational Mab only, were set up and processed under identical experimental conditions. Lane 1: VLPs incubated overnight without the Mab; lane 2: Mab incubated overnight; lane 3: VLPs incubated overnight with the H6.B10.5 conformational Mab; lane 4: total cell extract from the JSC310-6L1epi strain; lane 5: total cell extract from the JSC310-16L1epi strain. Arrows indicate a conformational Mab-derived band (A), the L1 bands (B) and a protein A Sepharose-derived band (C).

FIG. 9 depicts a characterization of sera derived from mice immunized with HPV-6, HPV-16 and mosaic VLPs. (A) Comparable amounts of HPV-6 (lanes 1), HPV-16 (lanes 2) and mosaic VLPs (lanes 3) were separated on SDS-PAGE and immunoblotted with antisera from mice immunized with HPV-6 VLPs (a) HPV-16 VLPs (b) and mosaic VLPs (c). (B) Comparable amounts of HPV-6 and HPV-16 VLPs were dot-blotted under denaturing and reducing (D) and nondenaturing and nonreducing (N) conditions and incubated with the S16 antiserum of a mice immunized with mosaic VLPs.

Detailed Description of the Invention

To test the possibility of inducing antibodies against multiple HPV types, we have generated a recombinant yeast diploid strain that coexpresses the HPV-6 and HPV-16 L1 and L2 genes. HPV-6/16 mosaic VLPs were purified from the cell lysate and used as antigens to immunize mice. The data presented below supports the formation of mosaic VLPs comprising all four proteins. The immunoprecipitation experiment strongly suggests that the CsCl purified VLPs represent the result of a reciprocal interaction of the two L1 proteins, rather than the simple coexistence of different VLP types. The fact that the L2 proteins are present in the same CsCl fractions favors the hypothesis that they are incorporated into the VLPs as well, since the L2 protein alone does not band in a CsCl gradient at the same density as L1 VLPs (22). Further, antisera able to recognize conformational epitopes of both L1 proteins were obtained. Although it remains to be confirmed that the immune response elicited by HPV-6/16 VLPs can neutralize the two viruses, the data herein supports using mosaic VLPs to immunize against a broader spectrum of virus types.

A yeast expression system as herein disclosed is preferred. Different laboratories have observed that a *Saccharomyces cerevisiae* expression system can be successfully used to easily purify PV VLPs (14, 18) which are highly efficient at inducing a protective immune response in animal models (20). Yeast-expressed VLPs are able to elicite a specific immune response not only at systemic but also at mucosal level. Lowe et al. have reported the generation of IgG neutralizing antibodies in the sera and genital secretions of African green monkeys immunized intramuscularly with HPV-11 VLPs, adsorbed to aluminum adjuvant (27). Greer et al. have observed the induction of anti-L1 specific IgG and IgA antibodies in the sera and genital secretions of mice immunized intranasally with HPV-6 VLPs, adjuvanted either with *E. coli* heat-labile enterotoxin (LT) or with a LT-derived non toxic mutant (14). Further, yeast expression affords the potential to scale-up to thousands of liters at relatively low cost and many yeast-derived products for human use are already market approved due to their safety.

To express the HPV-6 and HPV-16 L1 and L2 genes in the same yeast cell, we generated a *S. cerevisiae* diploid strain by mating two haploid strains, each

expressing two of the four capsid proteins. In order to obtain expression of the heterologous genes under identical culture conditions, each of them was cloned into the same expression cassette based on the ADH2/GAP glucose-repressible hybrid promoter and the $T_{MF\alpha}$ transcriptional termination sequence. The HPV-6 and HPV-16 L1 proteins were expressed by means of the episomal expression vector pBS24.1. Expression of the HPV-6 and HPV-16 L2 proteins was instead obtained by cloning the expression cassette into an integrative plasmid suitable for insertion into the *lys*2 locus of the haploid strain genome (Fig.4b). As a consequence of this cloning strategy, the L1 and L2 gene copy numbers in the haploid strains were different and this resulted in higher expression levels of the L1 proteins. This should resemble the ratio of L1 to L2 observed in native HPV virions, which has been estimated over a range from 5:1 to 30:1 (25). Table 1 lists the parental yeast strains used, the two recombinant haploid strains obtained and the diploid strain resulting from the mating.

TABLE 1. List of parental and recombinant yeast strains with genotypes and HPV expressed genes

Yeast strain	Genotype	Episomal HPV gene	Integrated HPVgene
JSC310	*MAT*a *leu*2-3 *ura*3-52 *prb*1-1122 *pep*4-3 *prc*1-407 *adr*1::DM15 *cir°*		
AB110	*MAT*α *leu*2-3-112 *ura*3-52 *pep*4-3 *his*4-580 *cir°*		
JSC310-6L1epi	*MAT*a *prb*1-1122 *pep*4-3 *prc*1-407 *adr*1::DM15 *cir°*	6L1	
JSC310-16L1epi	*MAT*a *prb*1-1122 *pep*4-3 *prc*1-407 *adr*1::DM15 *cir°*	16L1	
JSC310-6L2epi	*MAT*a *prb*1-1122 *pep*4-3 *prc*1-407 *adr*1::DM15 *cir°*	6L2	
JSC310-16L2epi	*MAT*a *prb*1-1122 *pep*4-3 *prc*1-407 *adr*1::DM15 *cir °*	16L2	
JSC310-6L2int	*MAT*a *leu*2-3 *ura*3-52 *prb*1-1122 *lys*2 *pep*4-3 *prc*1-407 *adr*1::DM15 *cir°*		
6L2			
AB110-16L2int	*MAT*α *leu*2-3-112 *ura*3-52 *pep*4-3 *lys*2 *his*4-580 *cir°*		16L2
JSC310-16L1/6L2	*MAT*a *prb*1-1122 *lys*2 *prc*1-407 *pep*4-3 *ade*2 *adr*1::DM15 *cir°*	16L1	
6L2			
AB110-6L1/16L2	*MAT*α *pep*4-3 *lys*2 *his*4-580 *cir°*	6L1	16L2
AB/JSC-4L	*MAT*a/*MAT*α *PRB*1/*prb*1-1122 *lys*2/*lys*2 *PRC*1/*prc*1-407 *pep*4-3/*pep*4-3	6L1-16L1	6L2-
16L2			
	*HIS*4/*his*4-580 *ADR*1/*adr*1::DM15 *cir°*		

As used herein, the term "mosaic VLP" refers to a VLP comprising capsid proteins from more than one type of virus. VLPs which result from intra- and/or inter-capsomeric association of the proteins are included.

As used herein, the term "type" in reference to viruses includes viruses (animal and plant) within the same family, group, or genus as well as viruses in different families, groups, or genuses.

As used herein, the term "non-integrative" in reference to a vector indicates that the vector does not integrate into the host DNA.

Yeast strains. The *Saccharomyces cerevisiae* haploid strains used were JSC310 (MATa, *leu*2-3, *ura*3-52, *prb*1-1122, *pep*4-3, *prc*1-407, *adr*1::DM15, *cir °*) (17) and AB110 (MATα, *leu*2-3-112, *ura*3-52, *pep*4-3, *his*4-580, *cir °*) (43), provided by Vicky Hines (Chiron Corporation, Emeryville, CA, USA).

Monoclonal and polyclonal antibodies. The H6.C6 and H16.H5 monoclonal antibodies (Mabs), which bind to denatured HPV-6 and HPV-16 L1 proteins, respectively, in addition to the H6.B10.5 and H16.V5 Mabs, specific for HPV-6 and HPV-16 intact VLPs, have been reported by Christensen et al. (8, 9). For Western blot analysis, these Mabs were used at 1:3000 dilution with a 4°C overnight incubation. HPV-16 L2 rabbit antiserum was a gift of Lutz Gissmann (DKFZ, Heidelberg, Germany), while HPV-6 L2 rabbit antisera were kindly provided by Denise Galloway (Fred Hutchinson Cancer Research Center Seattle, Washington) and Robert C. Rose (University of Rochester, NY). All the antisera were used at 1:3000-5000 dilution with a 4°C overnight incubation. Anti-rabbit and anti-mouse peroxidase-conjugated antibodies were from Biosource International (Camarillo, CA) and were used at 1:5000 dilution at room temperature for 1.5 hours.

Example 1

HPV type-specific detection of capsid proteins expressed in yeast.

A single yeast strain which could express the four HPV-6 and HPV-16 L1 and L2 capsid proteins was prepared. A necessary tool in achieving this was the availability of antibodies which reacted specifically or preferentially with the L1 or the L2 protein of only one HPV type. The HPV-6 and HPV-16 L1 and L2 genes were cloned in the episomal vector pBS24.1 (see Example 2 below) and expressed in the *S. cerevisiae* strain JSC310 to test the type specificity of the available antibodies. Fig. 3 shows the results of a Western blot analysis of total cell extracts prepared from the recombinant strains incubated with specific anti-HPV-6 (a) or HPV-16 (c) L1 Mabs and with HPV-6 (b) or HPV-16 (d) L2 antisera. In all cases HPV type-specific bands were detected, although a weak cross-reactivity could be seen for both the L2 antisera. While the HPV-6 and HPV-16 L1 Mabs identified proteins with the expected molecular weight of about 55 kilodalton (kDa), the L2 proteins, as previously reported (11, 12), showed an electrophoretic mobility corresponding to approximately 72-75 kDa, instead of the 55 kDa predicted on the basis of their amino acid sequences.

Example 2

Construction of recombinant plasmids

DNA fragments encoding the HPV proteins were obtained from available recombinant plasmids, either by restriction enzyme digestion or by PCR amplification (Expand High Fidelity PCR System, Boehringer Mannheim), and they were completely sequenced using an Applied Biosystem (Norvalk, CELLTECH, USA) model 373 DNA sequencer.

The episomal yeast expression vector pBS24.1, a yeast "shuttle" vector (17 and Philip J. Barr, Chiron Corporation, Emeryville, CA, USA), containing the leucine 2 (Leu2) and uracil 3 (Ura3) selectable genes was used. In this instance, it was obtained by digesting an available pBS24.1αt6E7 plasmid with Bam HI and Sal I . The pBS24.1αt6E7 plasmid was prepared for the yeast expression of the HPV-6E7 antigen in a secreted form.

The pBS-6L1 plasmid, expressing the HPV-6 L1 protein under the control of the alcohol-dehydrogenase-2-glyceraldehyde-3-phosphate-dehydrogenase (ADH2/GAP) glucose repressible promoter (J. Shuster, Chiron Corporation, Emeryville, CA, USA) and the mating type alpha factor gene transcriptional termination sequence ($T_{MF\alpha}$) was derived from the pBS24.1 plasmid as follows.

The plasmid pBS-6L1 is a yeast expression vector which contains the HPV-6L1 under the control of the ADH2\GAP promoter cloned into BAM HI and Sa1 I sites of the vector pBS24.1. The vector pBS24.1 contains the α-factor terminator, therefore an "expression cassette" for HPV-6 L1 is obtained. The "expression cassette" for HPV-6L1 consists of the following sequences fused together (from 5' to 3'): ADH2\GAP hybrid promoter, HPV-6L1 gene, and α-factor terminator. At the end of the cloning procedures the above "expression cassette" was obtained into the pBS24.1 (17). The vector pBS24.1 may be replicated both in *Escherichia coli* and in *Saccharomyces cerevisiae* since it contains PBR322 sequences (including the origin of replication and the ampicillin resistance gene) and the complete 2μ sequences (including the origin of replication). It also contains the yeast URA3 gene and the yeast LEU2 gene.

A summary of the construction of plasmid pBS24.1-A/G-6L1 is presented schematically in Figure 1. Due to the lack of suitable restriction sites, the fusion between the glucose repressible ADH2\GAP promoter and the L1 ORF has been obtained by means of recombinant PCR. The 1-563 bp segment of the hybrid promoter (1113 bp long) is derived from GAGαt6E7 plasmid whilst the 564-1113 bp are derived from PCR amplification of Gga plasmid (see below). The 1-115 bp segment of L1 sequence (1503 bp long) is derived from PCR amplification of the pAcC13-6L1 plasmid (Greer et al., *J. Clin. Microbiology*, 2058-2063, 1995 and Munemitsu et al., *Mol. Cell. Biol.*, 10:5977-5982, 1990), whilst the 116-1503 bp segment is derived from pAcC13-6L1 plasmid directly. The DNA sequence of HPV 6 is reported in Schwarz et al., *EMBO J.*, 2:2341-2348, 1983.

The GAGαt6E7 plasmid is a derivative of pGEM-3z (Promega) vector in which the following sequence was constructed (from 5' to 3'): ADH2\GAP promoter, an α-factor derived leader sequence, and the HPV-6E7 coding sequence. The GAGαt6E7 plasmid was digested with Bcl I and Xba I. The DH5α derived plasmid DNA could not be cut with Bcl I because the DH5α cells are dam+, but the Bcl I enzyme is inhibited by overlapping dam methylation; in order to obtain a Bcl I digestible DNA the plasmid was transformed in the dam- JM110 *E. coli* cells (Stratagene). The JM110 derived plasmid was digested with Bcl I and Xba I, the fragment containing the vector and the 5' half of the ADH2\GAP promoter was gel purified and set aside for further ligation.

The pAcC13-6L1 plasmid was digested with Xba I, the insert was gel purified and set aside for ligation. The Xba I insert consisted in the L1 sequence from bp 115 to the end of the sequence, including the stop codon.

The recombinant PCR is schematically represented in Figure 2. The sequences of the primers are listed below.

RP5 5'- ACTGATAGTTTGATCAAAGGGGCAAAACGTAGGGGC-3' SEQ ID NO:1

RP6 5'-GTCGCTAGGCCGCCACATGGTGTTTGTTTATGTGTG-3' SEQ ID NO:2

RP7 5'-AAACACACATAAACAAACACCATGTGGCGGCCTAGC-3' SEQ ID NO:3

RP8 5'-GCAGTCACCACCCTGTACAGGTGTATTAGTACACTG-3' SEQ ID NO:4

A first PCR was performed using the RP5 and RP6 primers and the Gga plasmid DNA as template. The Gga plasmid is a pGEM-3z plasmid derivative obtained in the context of the previous procedures for the HPV-6E7 cloning in yeast and contains the ADH2\GAP promoter. The goal of this first PCR was to obtain the 563-1113 bp portion (3' half) of the ADH2\GAP promoter. The RP5 primer overlapped a Bcl I site. A second PCR was performed using the RP7 and RP8 primers and the pAcC6L1 (Greer et al., 1995) plasmid as template. The goal of this second PCR was to amplify the 5' end of the L1 sequence from the initiation codon to the bp 543. The amplified fragment would contain an Xba I site at position 115. The RP6 and RP7 primers were designed in such a way that the 3' end of the first PCR product would anneal to the 5' end of the second PCR product. A third PCR was performed by mixing the first and second amplimers and the external primers RP5 and RP8. During this PCR a joining between first and second amplimers would happen and also an amplification of the joined product.

The expected 1126 bp product of the third PCR was predicted to consist in the 563-1113 (3' half) sequence of the ADH2\GAP promoter joined to the 1-530 (5' end) sequence of the HPV-6L1 ORF. The final PCR product would have a Bcl I site at the 5' end and an Xba I site in the L1 portion of the sequence at position 115. The third PCR product was digested with Bcl I and Xba I and gel purified. The fragment containing the pGEM-3z vector and the 5' half of the promoter coming from the Bcl I-Xba I digestion of the GAGαt6E7 plasmid was ligated with the Bcl I-Xba I digested recombinant PCR product and to the L1 insert coming from the Xba I digestion of pAcC13-6L1 plasmid.

After transformation into DH5α cells, several transformants were obtained. The miniprep DNAs from 14 transformants were digested using Eco RI. The Eco RI enzyme was chosen because by using this enzyme it has been possible to verify both the expected molecular sizes and the correct orientation of the 6L1 fragment. The

6L1 fragment had identical extremities (such as Xba I), therefore the probability for the fragment to assume an opposite orientation was 50%. By using Eco RI the plasmid DNA of the right clones should give two fragments, 2600 and 2700 bp long. The miniprep DNA of the n°8 clone gave a single band on a first gel but by running the gel much more was possible to resolve the 2600 and 2700 bp fragments. Also using Sph I it was possible to have a further indication that the clone n°8 was good. It was, thus, assumed that the clone n°8 contained the correct pGAG-6L1 plasmid consisting in the pGEM-3z vector containing the HPV-6 L1 sequence under the control of the ADH2\GAP promoter.

The ADH2\GAP-HPV-6L1 insert was excised from pGAG-6L1 plasmid by digesting with Bam HI and Sal I, the insert was gel purified and set aside for further ligation. The promoter-L1 fragment and the pBS24.1 vector were ligated and the product of the reaction was transformed into DH5α cells. The miniprep DNAs from 5 transformants were analyzed by digesting the Bam HI and Sal I and the clones A, B, C, and E were selected as good clones exhibiting the right molecular weight pattern.

A clone was transformed in JSC310 strain of *Saccharomyces cerevisiae* by means of electroporation and the cells were plated on URA- plates. Selected transformants were picked from URA- plates and streaked on LEU- plates. Single colonies from LEU -plates were inoculated in LEU- medium. Four clones grown in LEU- medium were reinoculated in YEPD medium. Cell pellets from the four JSC310-6L1 clones, A, B, C and D were frozen at -20°C after 24 and 48 hours of growth in YEPD medium on purpose to check L1 protein expression. Glycerol batches of the four clones were stored at -80°C.

The 6L1 yeast cell pellets were glass beads extracted, soluble and insoluble extracts were separated by means of centrifugation and prepared for SDS-PAGE analysis. Extracts from a strain not containing the pBS-6L1 plasmid (JSC310 cells transformed with pAB24 vector) were also prepared as a negative control. In Coomassie strained gel and in western immunoblot an induced band exhibiting the expected molecular weight was visible. A comparison of the HPV-6L1 expressed in

the yeast JSC310 strain and the same antigen expressed in insect cells showed that the two antigens have similar molecular weight.

The DNA portion of the L1 gene deriving from recombinant PCR (bp 1-115) has been sequenced using the following primer:

5' TAGTTTTAAAACACCAA 3' SEQ ID NO:12.

The primer annealed at the 3' end of the ADH2\GAP promoter, at position -37from the L1 start codon. The pGAG-6L1 plasmid (pGEM-3z containing the ADH2\GAP promoter fused to the L1 sequence) was used as template. By sequencing it was established that no errors occurred during the recombinant PCR manipulations nor in the cloning steps.

To construct the YIpAde integrative plasmid, a 1,059 bp *Xba*I genomic DNA fragment of the *S. cerevisiae* adenine 2 gene (Ade2) was amplified by using the PCR oligonucleotide primers 5'AdeE (5'-GCGGCGAATTCTAGAACAGTTGGTATATTAG-3' SEQ ID NO:5, inserting an *Eco*RI site) and 3'AdeP (5'GCGGCCTGCAGGGTCTAGACTCTTTTCCATATA-3'SEQ ID NO:6, inserting a *Pst*I site). The amplified DNA fragment was cloned into plasmid pUC8 digested with *Eco*RI and *Pst*I and the *Xba*I sites, included in the amplified DNA fragment, were used to excise the insert for yeast transformation. To obtain the integrative YIpLys-L2 expression plasmids, a 1,318 bp genomic DNA fragment of the *S. cerevisiae* lysine 2 (Lys2) gene was amplified by using the PCR oligonucleotide primers 5'LysE (5'-GCGGAATTCCACTAGTAATTACA-3'SEQ ID NO:7, inserting an *Eco*RI site) and 3'LysH (5'-GATGTAAGCTTCTACTAGTTGA-3'SEQ ID NO:8, inserting a *Hind*III site). The amplified DNA fragment was then inserted into pUC8 (derivatives readily available from commercial sources, e.g., Promega) digested with *Eco*RI and *Hind*III, generating a plasmid named YIpLys. A *Bam*HI DNA fragment from pSI3 vector (Isabel Zaror, Chiron Corporation, Emeryville, CA, USA, pBR322 backbone, ADH2/GAP promoter, SOD protein, and $T_{MF\alpha}$), including the ADH2/GAP promoter, the human superoxide dismutase (SOD) gene and the $T_{MF\alpha}$ transcriptional termination sequence, was cloned into the single *Bgl*II restriction site in the Lys2 gene sequence of YIpLys, obtaining a plasmid named YIpLys-SOD. The YIpLys-6L2 plasmid was derived from YIpLys-SOD

replacing the *Nco*I-*Sal*I DNA fragment encoding the SOD gene with the *Nco*I-*Sal*I DNA fragment from pGEM3z-6L2 (Kent Thudium, Chiron Corporation, Emeryville, CA, USA) encoding the HPV-6b L2 open reading frame (ORF). To construct the YIpLys-16L2 plasmid, the L2 gene was amplified from the cloned HPV-16 genomic DNA (kindly provided in this instance by Dennis J. McCance, University of Rochester, NY) by using the PCR oligonucleotide primers DT-5'L2 (5'-CGACACAAACGTTCTGCAA-3'SEQ ID NO:9) and DT-3'L2 (5'-ATTAGTCGACCTAGGCAGCCAAGAGACATC-3'SEQ ID NO:10), including the translation termination codon and a *Sal*I site. The DNA fragment obtained was digested with *Sal*I and cloned into YIpLys-SOD from which the SOD coding sequence had been removed by digestion with *Nco*I, filling-in with Klenow enzyme and digestion with *Sal*I.

The pBS-6L2 and pBS-16L2 episomal expression plasmids were obtained by replacing a *Sac*I-*Sal*I DNA fragment from pBS-6L1, including part of the ADH2/GAP promoter and the entire HPV-6b L1 ORF, with *Sac*I-*Sal*I DNA fragments, derived from either YIpLys-6L2 or YIpLys-16L2, including the corresponding promoter region and the L2 ORF.

To construct the pBS-16L1 episomal expression plasmid, the L1 gene was amplified from cloned HPV-16 genomic DNA by using the PCR oligonucleotide primers DT-5'L1 (5'-TCTCTTGGCTGCCTAGTGAGGCCA-3' SEQ ID NO:11) and DT-3'L1 (5'-CTAGTAATGTCGACTTACAGCTTACGTTTTTTGCG-3'SEQ ID NO:12), comprising the translational termination codon and a *Sal*I site. The amplified DNA fragment was purified from agarose gel and cloned into blunt-ended pSI3 vector from which the SOD gene had been previously removed by digestion with *Nco*I and *Sal*I restriction enzymes and filling-in with Klenow enzyme. From this intermediate construct, a *Sac*I-*Sal*I DNA fragment, including part of the ADH2/GAP promoter and the HPV-16L1 ORF, was purified and used to replace the corresponding *Sac*I-*Sal*I DNA fragment in pBS-6L1.

Example 3

Generation of recombinant yeast strains

The strains JSC310-6L1epi (14), JSC310-16L1epi, JSC310-6L2epi and JSC310-16L2epi, expressing the four capsid proteins by means of episomal vectors, were obtained by transformation of the parental JSC310 strain with the expression plasmids pBS-6L1 (14), pBS-16L1, pBS-6L2 and pBS-16L2.

The JSC310-6L2int and the AB110-16L2int strains were obtained using the following experimental approach. Competent yeast cells were cotransformed with 5μg of *Eco*RI-*Hind*III digested YIpLys-6L2 or YIpLys-16L2 integrative plasmid and 1μg of pBS24.1 episomal vector to allow the selection of transformants. Different clones were tested for growth onto plates of minimal medium (MM) supplemented with α-adipate to select mutants with an inactivated Lys2 gene (49). Correct integration into the *lys*2 locus was verified by PCR analysis by using pairs of oligonucleotide primers complementary to sequences within the expression cassette and the genomic portion of the Lys2 gene. Among the colonies expressing the L2 protein, one was chosen, cured of the pBS24.1 plasmid and tested for the inability to grow in the absence of uracil and leucine. Introduction of the episomal L1 expressing vectors into these strains was carried out following two different strategies. AB110-16L2int was transformed with the pBS-6L1 expression plasmid and selection of transformants on MM plates without leucine and uracil allowed the isolation of the haploid strain AB110-6L1/16L2. The JSC310-6L2int strain was instead cotransformed with the pBS-16L1 expression vector and with the *Xba*I digested YIpAde integrative plasmid. Transformants grown on selective plates were plated on complete yeast extract-peptone medium (YEP) and allowed to grow at 30°C for 3-4 days until colonies (1-2%) developed a red color due to disruption of the *ade*2 locus (52). One of the clones, which showed correct integration into the *ade*2 locus by PCR and L1 and L2 expression by Western blot analysis, was designated JSC310-16L1/6L2.

Generation of the AB/JSC-4L diploid strain was obtained by mixing cultures, in YEP medium containing 5% glucose, of the two haploid strains, AB110-6L1/16L2 and JSC310-16L1/6L2. Selection of the AB/JSC-4L diploid strain required an

additional genetic marker in the haploid JSC310-6L2int strain. This was obtained inactivating the endogenous Ade2 gene by means of the integration plasmid represented in Fig.4a. Diploid cells were selected onto MM plates lacking histidine and adenine.

Expression of the four proteins in the haploid strains and in the strain resulting from their mating was evaluated by Western blot analysis. Fig.5 shows the results of such experiments demonstrating that both the haploid strains AB110-6L1/16L2 (a and d, lanes 1) and JSC310-16L1/6L2 (b and c, lanes 2) expressed the heterologous genes and that the expression of all four proteins was stably maintained in the resulting AB/JS-4L diploid strain (a, b, c and d, lanes 3).

Example 4

Preparation of VLPs

Parental yeast strains were grown in complete YEP medium. Strains transformed with episomal vectors were first cultured in leucine-deficient MM medium with 4% glucose until they reached midlog phase. Expression of the genes under the control of the ADH2/GAP glucose-repressible promoter was induced by diluting these cultures 1:50 into YEP complete medium and culturing the cells at 30°C for 2-3 days. Total cell extracts were prepared from 3.5 optical densities (OD) of yeast cell cultures grown to approximately OD_{600}=20. Cells were lysed with a 10 minute incubation on ice in 0.24 N NaOH and 0.96% β-mercaptoethanol, followed by trichloroacetic acid (TCA) precipitation, ice cold acetone washing and final suspension of the protein pellet in 100 µl of protein loading buffer. To carry out dot-blot experiments where preservation of L1 conformation was necessary, yeast cells were collected, washed, suspended in phosphate-buffered saline (PBS, pH 7.5) and disrupted by vortexing five times for 1 minute in the presence of glass beads (425-600 µm, Sigma).

Frozen yeast cell pellets were thawed in buffer containing 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, 2 mM $MgCl_2$ and 1 mM EGTA (#E3889, Sigma Chemical Co.) and Complete™Protease Inhibitors (#1-697-498, Boehringer Mannheim). Cells were disrupted by vortexing twice for 10 minutes, with a 5 minute interval on ice, in the

presence of glass beads (0.5 ml beads per ml of cell suspension) using a VWRbrand Multi-tube vortexer (VWR Scientific Product). Cellular debris was removed by a 20 minute centrifugation at 2000 x g. The supernatants were then centrifuged through a 40% (w/w) sucrose cushion (2 hour centrifugation at 100,000 x g). The resulting pellets were suspended in PBS, applied to a pre-formed CsCl gradient (1.17-1.57 g/ml) and centrifuged for 24 hours at 285,000 x g. The gradients were fractionated and aliquots from each fraction were subjected to Western blot analysis with type-specific anti-L1 and anti-L2 antibodies. Peak fractions were pooled and dialyzed against PBS. Total protein concentration was determined by BCA™ Protein Assay Reagent (#23225, Pierce Chemicals).

Example 5

Characterization of VLPs

Proteins were analyzed by denaturing sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10% polyacrylamide) and Western blotting onto nitrocellulose membrane (pore size 0.45 μm, MSI, Westborough, MA USA) according to standard protocols. Dot-blot analysis of denatured and reduced VLPs was carried out boiling the protein samples for 5 minutes in the presence of dithiothreitol (DTT) before applying them to nitrocellulose filters using a bio-dot apparatus (Biorad). When native VLP structure had to be maintained, VLPs in PBS were applied to the membrane without boiling and in the absence of DTT. Reaction with HPV-specific antibodies was detected using the Enhanced Chemiluminescence (ECL) Western blotting reagent (Amersham) and Hyperfilm ECL (Amersham).

Specifically, the cell extract from the diploid strain was subjected to CsCl gradient sedimentation and aliquots of the collected fractions were boiled in the presence of DTT and blotted in duplicate onto nitrocellulose filters. The filters were incubated with anti-HPV-6 and anti-HPV-16 specific Mabs which react with denatured L1 (8, 9), revealing that the two L1 proteins were enriched in the same fractions (Fig. 6A, a and c). The dot-blot experiment was repeated without denaturing and without reducing the protein samples and using anti-HPV-6 and HPV-16 L1 specific Mabs which were previously reported to react exclusively with intact VLPs

in enzyme-linked immunosorbent assay (ELISA) experiments (8, 9). The result obtained confirmed that the two conformationally dependent Mabs were able to recognize the L1 proteins which copurified in the CsCl fractions (Fig 6A, b and d). As expected, the two Mabs reacted specifically with HPV-6 and HPV-16 control VLPs only under nondenaturing and nonreducing conditions (Fig. 6A, e). Western blot analysis of fraction 5 confirmed that both HPV-6 and HPV-16 L2 proteins were also present (Fig. 6B, a and b). Estimation of the refractive index of the identified protein peak gave a value of 1.29-1.3 mg/ml. EM analysis of the enriched fraction revealed the presence of VLPs which appeared to be similar to control VLPs formed by either HPV-6 or HPV-16 L1 (Fig.7).

To evaluate whether the HPV-6 and HPV-16 L1 proteins could interact and assemble into mosaic VLPs, we performed immunoprecipitation experiments using CsCl banded VLPs and the specific anti-HPV-6 L1 conformationally dependent Mab H6.B10.5 (9). Approximately 1 μg of CsCL banded VLPs were diluted with PBS and incubated with the conformationally dependent anti-HPV-6 L1 Mab H6.B10.5 (1:1000 dilution) overnight at 4° C with gentle shaking. The immune complexes were collected with Protein A Sepharose CL-4B (Pharmacia Biotech), washed 4 times with 1 ml PBS, suspended in sample buffer, boiled for 5 minutes, subjected to SDS-PAGE and analyzed by Western blot using anti-HPV-6 and anti-HPV-16 L1 Mabs. The Western blot carried out on the immunoprecipitates using type-specific anti-L1 Mabs (Fig. 8) identified three major bands: (A) was a Mab-derived band, since it could be also observed when the conformational Mab was immunoblotted with the anti-mouse antibody; (B) was a band that appeared only when the VLPs were incubated with the conformational anti-HPV-6 L1 Mab (lanes 3), identifying specifically immunoprecipitated proteins with an electrophoretic mobility corresponding to that of HPV-6 L1 (a, lane 4) and HPV-16 L1 (b, lane 5); (C) was a resin-derived band that was also detected when an aliquot of protein A Sepharose was suspended in PBS and immunoblotted with the anti-mouse antibody. Bands (B) were not visible when the immunoprecipitation was carried out using an unrelated Mab Similarly, HPV-16 L1 could not be detected when HPV-6 and HPV-16 VLPs were mixed and immunoprecipitated.

Example 6

Mouse immunization with VLPs.

To investigate whether HPV-6/16 mosaic VLPs were able to induce an immune response directed against both HPV types, groups of mice were immunized subcutaneously with HPV-6, HPV-16 and mosaic VLPs and the sera were tested after the third immunization. Six week old female Balb/c mice were injected subcutaneously with 20 μg of the following purified antigens: (I) HPV-6 VLPs, (ii) HPV-16 VLPs, (iii) HPV-6/16 VLPs. All the antigens were administered with equal volume of MF59 adjuvant (30). A group of control mice was injected only with MF59. The mice were boosted with 15 μg of the respective antigen at week 3 and 10 μg at week 5. Serum samples were collected on day 12 after the final booster and assayed for capsid protein specific antibodies.

Figure 9A shows the result of the Western blot carried out with the three types of denatured VLPs incubated with three sera, each representative of the different groups of immunized mice. While the reactivity of the sera from mice immunized either with HPV-6 or HPV-16 VLPs was predominantly type-specific (Fig. 9A, a and b), the serum from mouse 16 (S16), immunized with HPV6/16 VLPs, reacted against both HPV-6 and HPV-16 L1 (Fig. 9A, c). To analyze whether the immune response was also directed against conformational epitopes of the L1 proteins, equal amounts of either HPV-6 or HPV-16 VLPs were blotted under denaturing and nondenaturing conditions and incubated with the S16 antiserum. Figure 9B shows that the signal was significantly lower when the samples were denatured and reduced, suggesting that conformational antibodies had been elicited.

The foregoing examples are meant to illustrate the invention and are not to be construed to limit the invention in any way. Those skilled in the art will recognize modifications that are within the spirit and scope of the invention. All references cited herein are hereby incorporated by reference in their entirety.

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What is claimed is:

1. A virus-like particle (VLP) comprising capsid proteins from at least two types of viruses.
2. The VLP of claim 1 wherein said viruses are animal viruses.
3. The VLP of claim 2 wherein said viruses are human viruses.
4. The VLP of claim 3 wherein said viruses are different types of Human Papilloma Virus (HPV).
5. The VLP of claim 4 wherein said types of HPV are types 6 and 16.
6. The VLP of claim 4 or 5 wherein the capsid proteins comprise the major capsid protein late 1 (L1).
7. The VLP of claim 4 or 5 wherein the capsid proteins comprise the minor capsid protein late 2 (L2).
8. The VLP of claim 4 or 5 wherein the capsid proteins comprise L1 from one virus type and L2 from a second virus type.
9. The VLP of claim 6, further comprising the L2 capsid protein.
10. A composition comprising the VLP of claim 1.
11. The composition of claim 10 wherein the VLP is purified for immunization.
12. The composition of claim 11 wherein the VLP comprises the VLP of claim 9.
13. The composition of claim 12, further comprising an adjuvant.
14. The composition of claim 13 wherein the adjuvant is MF59.
15. A method for producing VLPs comprising capsid proteins from at least two types of viruses, said method comprising

a) cloning said capsid proteins into expression cassettes comprising the same promoters and termination sequences; and

b) expressing said cassettes in the same host cell.

16. The method of claim 15 wherein the host cell is a yeast cell.
17. The method of claim 16 wherein the yeast is *Saccharomyces cerevisiae*.
18. The method of claim 15 wherein said viruses are different types of HPV.
19. The method of claim 18 wherein said types of HPV are type 6 and 16.
20. The method of claim 15 or 18 wherein the capsid proteins comprise L1.

21. The method of claim 15 or 18 wherein the capsid proteins comprise L2.

22. The method of claim 15 or 18 wherein the capsid proteins comprise L1 from one virus type and L2 from a second virus type.

23. The method of claim 20, further comprising L2 capsid proteins.

24. The method of claim 23 wherein said L1 protein expression cassettes are cloned into non-integrative vectors, and said L2 proteins expression cassettes are cloned into integrative vectors.

25. The method of claim 24 wherein the non-integrative vector is pBS24.1.

26. The method of claim 24 wherein the integrative vector is pUC8.

27. A host cell comprising vectors for expressing capsid proteins from at least two types of viruses.

28. The host cell of claim 27 wherein said viruses are different types of HPV.

29. The host cell of claim 28 wherein said types of HPV are types 6 and 16.

30. The host cell of claim 29 wherein said capsid proteins comprise L1.

31. The host cell of claim 29 wherein said capsid proteins comprise L2.

32. The host cell of claim 27 or 29 wherein said capsid proteins comprise L1 from one virus type and L2 from a second virus type.

33. The host cell of claim 30, further comprising L2 capsid proteins.

34. The host cell of claim 33 wherein said host cell is a diploid cell.

35. The host cell of claim 27 or 34 wherein said host cell is yeast.

36. The host cell of claim 35 wherein said yeast is *Saccharomyces cerevisiae.*

37. A method for inducing an immune response against more than one type of virus comprising administering the VLP of any of claims 1-5 or 9.

38. A method for inducing an immune response against more than one type of virus comprising administering the VLP of claim 6.

39. A method for inducing an immune response against more than one type of virus comprising administering the VLP of claim 7.

40. A method for inducing an immune response against more than one type of virus comprising administering the VLP of claim 8.

41. A method for expressing capsid proteins from at least two types of viruses, said method comprising

a) cloning said capsid proteins into expression cassettes comprising the same promoters and termination sequences; and

b) expressing said cassettes in the same host cell.

42. The method of claim 41 wherein the host cell is a yeast cell.

43. The method of claim 42 wherein the yeast is *Saccharomyces cerevisiae*.

44. The method of claim 41 wherein said viruses are different types of HPV.

45. The method of claim 44 wherein said types of HPV are type 6 and 16.

46. The method of claim 41 or 45 wherein the capsid proteins comprise L1.

47. The method of claim 41 or 45 wherein the capsid proteins comprise L2.

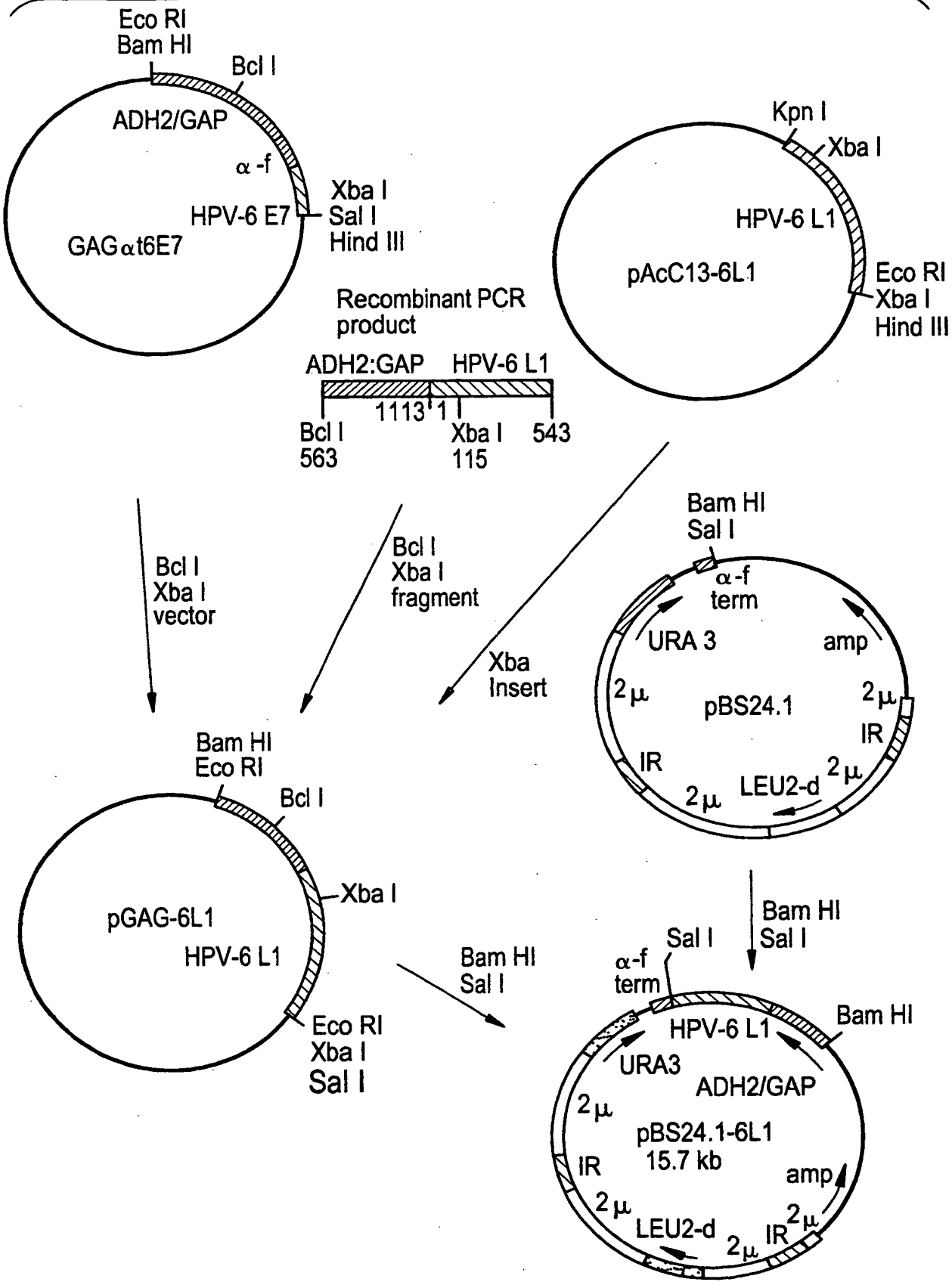
48. The method of claim 41 or 45 wherein the capsid proteins comprise L1 from one virus type and L2 from a second virus type.

49. The method of claim 46, further comprising L2 capsid proteins.

50. The VLP of claim 1 wherein said VLP induces an immune response against both types of viruses.

51. A composition comprising the VLP of claim 50.

FIG.1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Bam HI
Bcl I
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α -f
HPV-6 E7
Xba I
Sal I
Hind III
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Kpn I
Xba I
HPV-6 L1
pAcC13-6L1
Eco RI
Xba I
Hind III
Recombinant PCR product
ADH2:GAP
HPV-6 L1
1113
1
Bcl I
563
Xba I
115
543
Bcl I
Xba I
vector
Bcl I
Xba I
fragment
Xba
Insert
Bam HI
Sal I
α-f
term
URA 3
amp
2μ
pBS24.1
2μ
IR
IR
2μ
LEU2-d
2μ
Bam HI
Eco RI
Bcl I
Xba I
pGAG-6L1
HPV-6 L1
Eco RI
Xba I
Sal I
Bam HI
Sal I
Bam HI
Sal I
α-f
term
Sal I
HPV-6 L1
Bam HI
URA3
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LEU2-d
2μ
IR
2μ

FIG.2

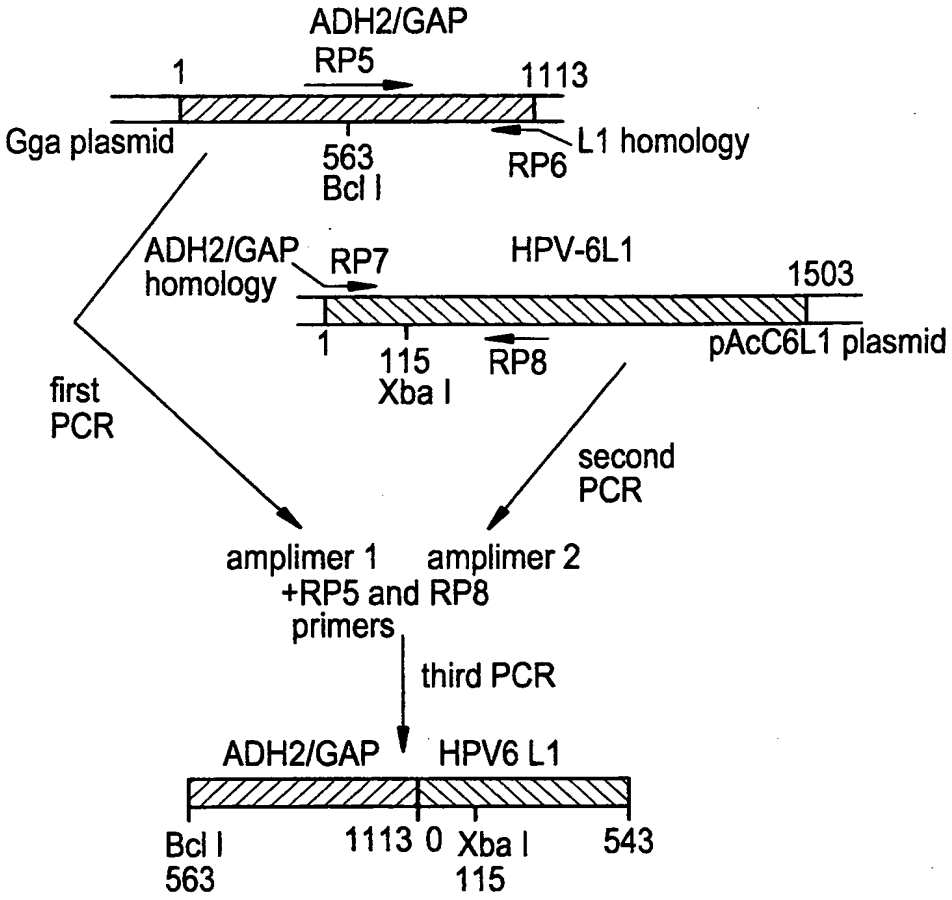


FIG.4A

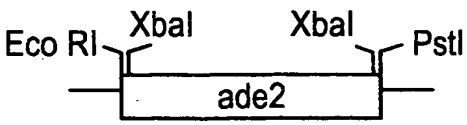


FIG.4B

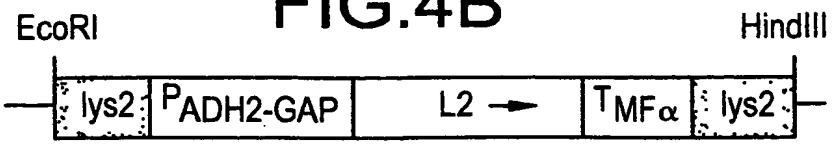


FIG. 3A

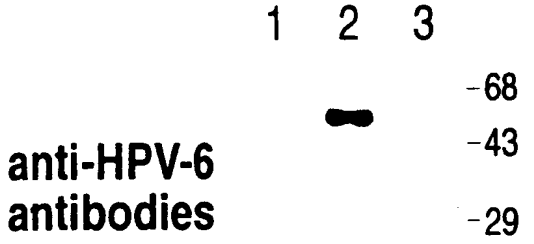


FIG. 3B

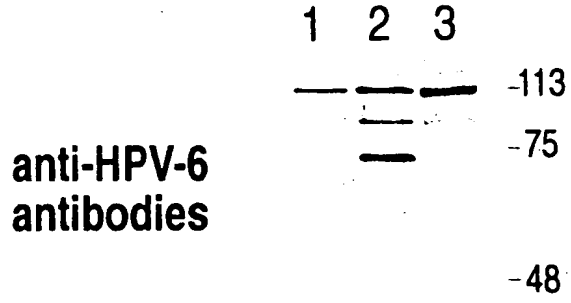


FIG. 3C

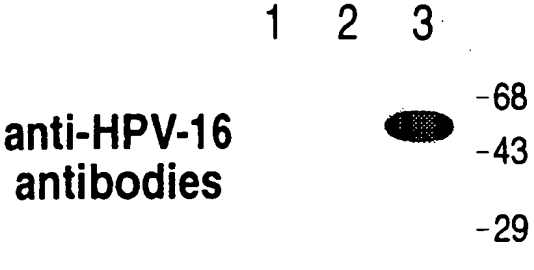


FIG. 3D

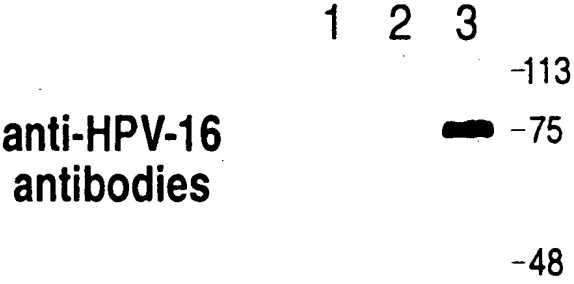


FIG. 5A

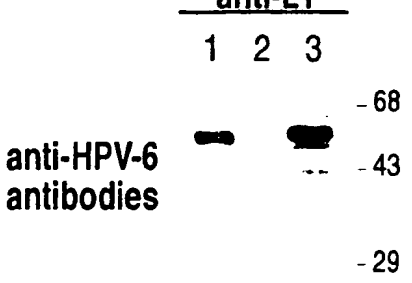


FIG. 5B

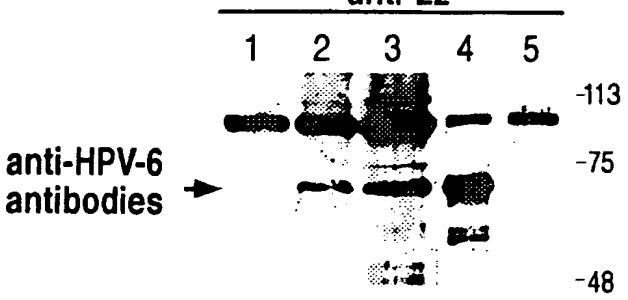


FIG. 5C

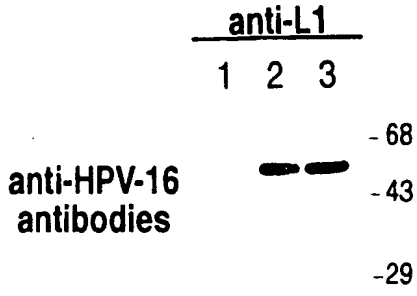


FIG. 5D

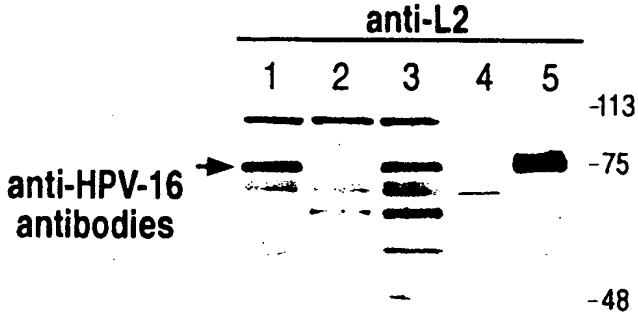


FIG. 6A

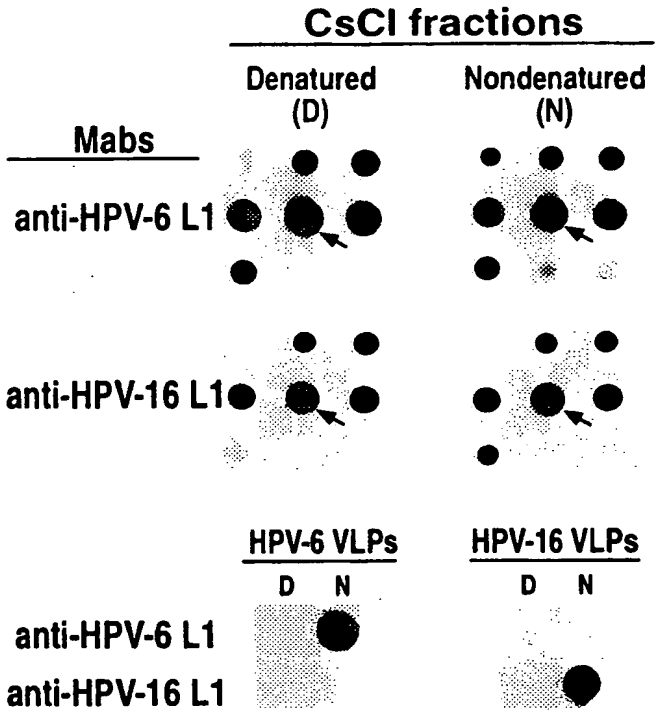

CsCl fractions
Denatured (D)
Nondenatured (N)
Mabs
anti-HPV-6 L1
anti-HPV-16 L1
HPV-6 VLPs
HPV-16 VLPs
D N
D N
anti-HPV-6 L1
anti-HPV-16 L1

FIG. 6B

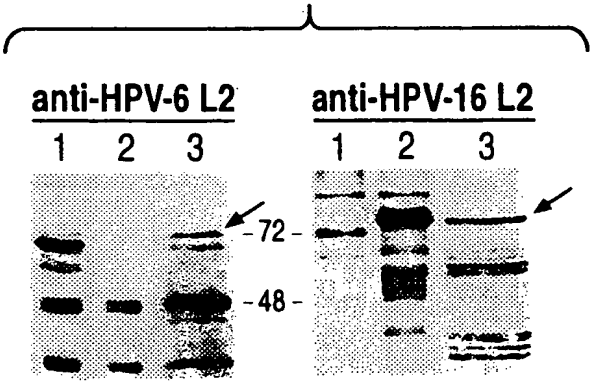

anti-HPV-6 L2
anti-HPV-16 L2
1 2 3
1 2 3
72
48

FIG. 7A

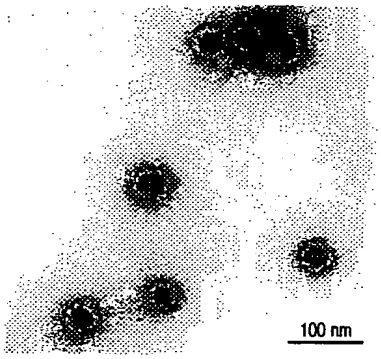


FIG. 7B

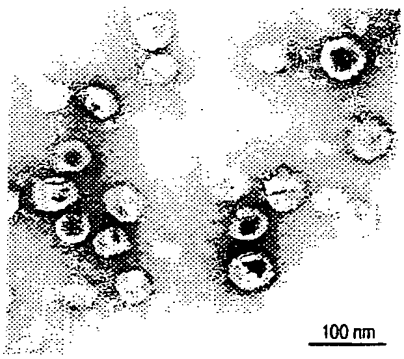


FIG. 7C

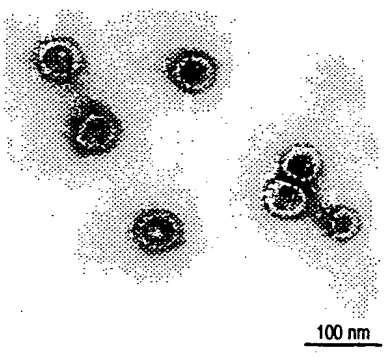


FIG. 8A

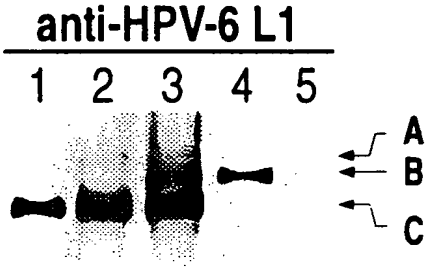


FIG. 8B

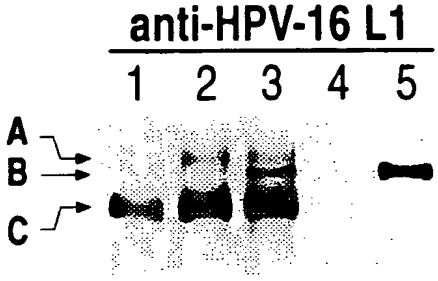


FIG. 9B

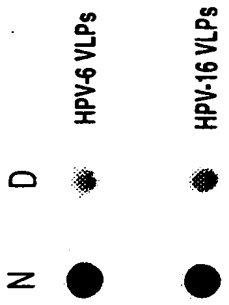
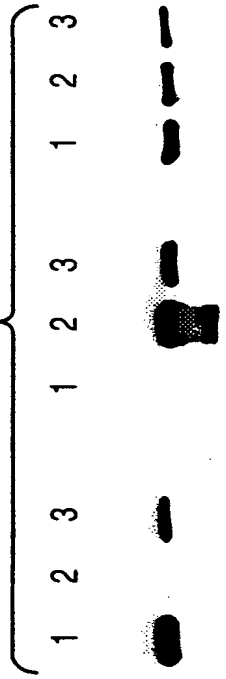


FIG. 9A



INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 99/18016

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/37 C07K14/205 A61K39/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 14564 A (LUDMERER STEVEN ;MERCK & CO INC (US)) 9 April 1998 (1998-04-09) examples 5,6	1-4,6,10
X	WO 96 05293 A (UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION ;BARALLE FRANCESCO) 22 February 1996 (1996-02-22) page 13, line 25 -page 17, line 10	1-3

-/—

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 February 2000	28/02/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Cupido, M

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 99/18016

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHANG C ET AL: "Phenotypic mixing between different hepadnavirus nucleocapsid proteins reveals C protein dimerization to be cis preferential" JOURNAL OF VIROLOGY, vol. 68, no. 8, August 1994 (1994-08), pages 5225-5231, XP002130379 AMERICAN SOCIETY FOR MICROBIOLOGY US cited in the application page 5228, left-hand column, last paragraph -page 5229, right-hand column, paragraph 1	1,2
A	HOFMANN K J ET AL: "Sequence conservation within the major capsid protein of human papillomavirus (HPV) type 18 and formation of HPV-18 virus-like particles in Saccharomyces cerevisiae" JOURNAL OF GENERAL VIROLOGY,GB,SOCIETY FOR GENERAL MICROBIOLOGY, READING, vol. 77, no. 3, March 1996 (1996-03), pages 465-468, XP000604634 ISSN: 0022-1317 the whole document	41-51

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Ir.. ..national application No.
PCT/US 99/ 18016

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 37-40 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern al Application No
PCT/US 99/18016

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9814564 A	09-04-1998	EP 0956349 A	17-11-1999
		US 5922588 A	13-07-1999
WO 9605293 A	22-02-1996	AT 402898 B	25-09-1997
		AT 154594 A	15-02-1997
		AU 3257495 A	07-03-1996
		EP 0775198 A	28-05-1997
		US 5932426 A	03-08-1999